REMARKS

Claims 1-11 and 18 and 19 are pending in the application. Claims 12-17 and 20 are withdrawn from consideration. For the Examiner's convenience, Applicants' remarks are presented in the order in which they were raised in the Examiner's Advisory Action. Reconsideration is respectfully requested.

Finality of previous Office Action

The Examiner has responded to Applicants' request to remove finality of the Office Action mailed dated November 7, 2003 by stating that "a previous Office Action was mailed on May 21, 2003." As Applicants noted previously, no such Office Action was received by the Applicants. An examination of the PAIR report (*see attached*) does not show any Office Action mailed by the PTO on May 21, 2003. The Examiner was requested to provide a copy of such Office Action of May 21, 2003 but none has been received by the Applicants to date. In the event the mailing of an Office Action of May 21, 2003 is in error, Applicants request reconsideration of request of remove finality of the Office Action mailed dated November 7, 2003.

In any event, Applicants are filing a Request for Continued Examination (RCE) to continue prosecution along with this response. However, Applicants are unable to respond to any issues that may have been raised in the "Office Action of May 21, 2003" as Applicants never received such an Office Action and the PTO's PAIR records do not provide any record of such Office Acton having been actually mailed.

Rejection under 35 U.S.C.§103

The Examiner notes that the rejection over Bramanti and Holt is based on on the antagonizing interaction between P. gingivalis and hemin by protoporphyrin IX and Zinc protoporphyrin (table 2). The Examiner further states that the Applicants arguments in their reponse filed May 7, 2004 are directed to the interaction between Omp26 protein and hemin and the Examiner contends that this interaction has no bearing on the grounds for rejection.

Applicants respectfully traverse. Table 2 of Bramanti and Holt merely shows that hemin analogs have an effect on [^{55}Fe]hemin uptake. The Examiner points to the fact that protoporphyrin IX and Zinc protoporphyrin IX show 57% and 55% inhibition, respectively, of ^{55}Fe uptake. This is not the same as showing that an antagonist of interaction between a molecule derived from a microorganism and having an HA2 domain and an HA2-binding motif on a porphyrin containing molecule (such as gingipain or *HHA* gene product or T1a protein) prevents the organism from acquiring iron heme or porphyrin. Such a conclusion cannot be reached from a reading of Bramanti and Holt (J. Bact. 175(22):7413-7420 (1993)) without application of improper hindsight based on the teachings of the present application.

Further commentary on hemin binding receptors were subsequently described in publications from the same laboratory (S.C. Holt) that authored the Bramanti reference.

The investigators reviewed their earlier findings with the conclusion that the outer membrane receptor expressed under hemin limitation in *Porphyromonas gingivalis* strain 381 (30 kDa; heated form 24 kDa) is a structural and functional homolog of the OMP26 receptor identified under hemin-limitation in strain W50 (Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of Porphyromonas gingivalis. *Periodontol 2000.* 1999 Jun; 20:168-238; see page 206).

Cyanogen bromide digestion of purified OMP30 from strain 381 yielded fragments amenable to sequence analysis. (Kim SJ, Chu L, Holt SC. Isolation and characterization of a hemin-binding cell envelope protein from Porphyromonas gingivalis. *Microb Pathog.* 1996 Jul;21(1):65-70). The reported sequences show close identity to the translated sequence of *hmuY*, a gene encoding a 15,547 Da TonB-dependent heme receptor identified by Simpson *et al.* (Simpson W, Olczak T, Genco CA. Characterization and expression of HmuR, a TonB-dependent hemoglobin receptor of Porphyromonas gingivalis. *J Bacteriol.* 2000 Oct; 182(20):5737-48). The authors report that *hmuR and hmuY* are cotranscribed and negatively regulated by iron.

Table 2 of the paper by Bramanti and Holt indicates that hemoglobin is not recognized by the Omp26 receptor. Studies by the inventors of the present application indicate that the HA2 receptor binds hemoglobin with a dissociation constant of 2.1nM compared to hemin and

protoporphyrin IX at 16nM. Hemoglobin, hemin and protoporphyrin IX all demonstrate competitive binding to HA2.

HmuR (*see* Simpson *et al.*) was subsequently demonstrated to have low affinity binding for protoporphyrin IX with the structure of the vinyl aspect of the porphyrin macrocycle exerting a major influence on recognition. (Olczak T, Dixon DW, Genco CA. Binding specificity of the Porphyromonas gingivalis heme and hemoglobin receptor HmuR, gingipain K, and gingipain R1 for heme, porphyrins, and metalloporphyrins. *J Bacteriol.* 2001 Oct;183(19):5599-608.). HmuY possibly has similar requirements. (*see* Simpson *et al.*). The interactions described in the Bramanti reference relate to the vinyl aspects of the molecules. In contrast to what is described by Bramanti, HA2 recognizes porphyrins in a manner independent of the vinyl aspect. (DeCarlo *et al.*).

Therefore, the Bramanti reference does not teach or suggest antagonizing "the interaction between a molecule derived from [a] microorganism having an HA2 domain and an HA2-binding motif on a porphyrin containing molecule" as specified in independent claim 1 (and similar terms in independent claims 9-11. Applicants respectfully request withdrawal of this ground for rejection under 35 USC 103.

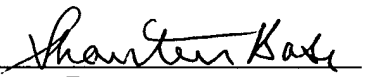
CONCLUSION

In light of the amendments and arguments set forth above, Applicants earnestly believe that they are entitled to a letters patent and respectfully request the Examiner to expedite prosecution of this patent application to issuance. Should the Examiner have any questions, the Examiner is encouraged to telephone the undersigned.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 229752001500. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: December 7, 2004

Respectfully submitted,

By 

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Characterization and Expression of HmuR, a TonB-Dependent Hemoglobin Receptor of *Porphyromonas gingivalis*

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The gram-negative pathogen *Porphyromonas gingivalis* requires hemin for growth. Hemoglobin bound to haptoglobin and hemin complexed to hemopexin can be used as heme sources, indicating that *P. gingivalis* must have a means to remove the hemin from these host iron-binding proteins. However, the specific mechanisms utilized by *P. gingivalis* for the extraction of heme from heme-binding proteins and for iron transport are poorly understood. In this study we have determined that a newly identified TonB-dependent hemoglobin-hemin receptor (HmuR) is involved in hemoglobin binding and utilization in *P. gingivalis* A7436. HmuR shares amino acid homology with TonB-dependent outer membrane receptors of gram-negative bacteria involved in the acquisition of iron from hemin and hemoglobin, including HemR of *Yersinia enterocolitica*, ShuA of *Shigella dysenteriae*, HpuB of *Neisseria gonorrhoeae* and *N. meningitidis*, HmbR of *N. meningitidis*, HgbA of *Haemophilus ducreyi*, and HgpB of *H. influenzae*. Southern blot analysis confirmed the presence of the *hmuR* gene and revealed genetic variability in the carboxy terminus of *hmuR* in *P. gingivalis* strains 33277, 381, W50, and 53977. We also identified directly upstream of the *hmuR* gene a gene which we designated *hmuY*. Upstream of the *hmuY* start codon, a region with homology to the Fur binding consensus sequence was identified. Reverse transcription-PCR analysis revealed that *hmuR* and *hmuY* were cotranscribed and that transcription was negatively regulated by iron. Inactivation of *hmuR* resulted in a decreased ability of *P. gingivalis* to bind hemoglobin and to grow with hemoglobin or hemin as sole iron sources. *Escherichia coli* cells expressing recombinant HmuR were shown to bind hemoglobin and hemin. Furthermore, purified recombinant HmuR was demonstrated to bind hemoglobin. Taken together, these results indicate that HmuR serves as the major TonB-dependent outer membrane receptor involved in the utilization of both hemin and hemoglobin in *P. gingivalis*.

The ability of a pathogen to scavenge essential nutrients within a particular environmental niche in the host is essential for the initiation and the establishment of an infection. Of these essential nutrients, iron plays a crucial role. Within the human host, the majority of iron is found in the form of heme proteins, including hemoglobin, or ferritin. Due to the abundance of heme proteins in the host, they are a valuable source of iron for bacterial pathogens. As a consequence, pathogenic organisms have developed diverse mechanisms for the acquisition of heme under the iron-limiting environment of the host (1, 9, 10, 12, 15, 19, 28, 40). The best-described system by which gram-negative bacteria acquire heme involves direct binding of free heme or heme proteins to specific outer membrane receptors (9). Energy for the transport of iron and/or heme ligands via these specific heme and hemoglobin receptors across the outer membrane into the periplasmic space is dependent on TonB, in association with the ExbB and ExbD proteins (5, 30). Recently, an additional system for the acquisition of heme involving an extracellular heme binding protein that functions to capture and shuttle heme to a specific outer membrane receptor has been described. In *Serratia marcescens*, the secreted protein HasA extracts heme from either hemopexin-heme or hemoglobin and delivers it to the outer membrane receptor HasR (17). Similar systems have been described in *Haemophilus influenzae* and *Pseudomonas aeruginosa* (10, 23).

Porphyromonas gingivalis, the etiological agent of adult periodontal disease, requires iron in the form of hemin for growth (13, 14) due to its inability to synthesize protoporphyrin IX, which it requires as the prosthetic group of cytochrome *b*. The latter serves as an electron sink during amino acid fermentation (8). Hemoglobin bound to haptoglobin and hemin complexed to hemopexin can be used as iron sources by *P. gingivalis*, indicating that this microorganism has a mechanism for removing the hemin from these host iron-binding proteins (4). In addition, *P. gingivalis* is capable of utilizing transferrin, found in serum, and lactoferrin, found on mucosal surfaces, for growth (13, 14). The characteristic black pigmentation produced by *P. gingivalis* colonies is due to the accumulation of μ-oxo dimers of hemin on the cell's surface (37). We have previously determined that *P. gingivalis* is capable of transporting the intact hemin molecule into the cell by an energy-dependent process (14). The energy dependence of hemin transport in *P. gingivalis* suggests that a TonB analog may function to transduce energy for the transport of hemin.

Hemin binding by *P. gingivalis* appears to occur through both high- and low-affinity receptors (13), and recent studies suggest that a common pathway may be utilized for the transport of hemin and hemoglobin (13); however, little is known regarding the specific *P. gingivalis* receptors for either ligand's binding. Hemin-binding proteins either induced by hemin limitation (4) or repressed by excess of this compound (38) have been described, but their role in hemin transport has not been further defined. Recently, two *P. gingivalis* TonB-dependent receptors, HemR and Tla, have been described (1, 19). The *hemR* gene from *P. gingivalis* 53977 exhibits homology to genes involved in iron acquisition in other bacterial species; however, conclusive evidence for the role of HemR in iron uptake from hemin or hemoglobin has not been reported (19). The Tla protein is

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
P. gingivalis		
A7436	Wild type	Lab collection
381	Wild type	Lab collection
W50	Wild type	T. van Dyke, Boston University, Boston, Mass.
ATCC 33277	Wild type	Lab collection
ATCC 53977	Wild type	P. Baker, Bates College, Lewiston, Maine
WS1	A7436, *hmuR::erm*	This study
E. coli		
DH5α	*recA1 lacZYA-argF supE44*	Promega
TOP10F'	F' [*lacI*^{q} Tn*10*(Tet^r)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* (*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
BL21(DE3)pLysE	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)pLysE (Cam^r)	Invitrogen
Plasmids		
pGEM3z(−)	Amp^r	Promega
pWS1	pGEM3z(−) containing 485-bp N terminus of *hmuR* and the *B. fragilis ermF* cassette within the *Pst*I site of the *hmuR* gene	This study
pCRT7/CT-TOPO	Amp^r	Invitrogen
pTO1	pCRT7/CT-TOPO containing the *hmuR* gene without the signal peptide sequence	This study
pTO2	pCRT7/CT-TOPO containing the *hmuR* gene with the signal peptide sequence	This study

required for growth of *P. gingivalis* with low levels of hemin; however, its role as a specific hemin receptor has not been defined.

Although previous studies have documented the ability of *P. gingivalis* to utilize hemoglobin as a sole iron source, receptors involved in the binding of this compound to the *P. gingivalis* cell have not been identified. Recent studies have reported that the lysine- and arginine-specific gingipains Kgp and HRgpA (31) can bind and subsequently cleave hemoglobin (11, 24; Sroka et al., submitted; C. A. Genco, A. E. Sroka, and J. Potempa, unpublished data). It is not clear which part of the Kgp complex participates in hemoglobin binding, since reports indicate that either the catalytic domain or the hemagglutinin domain is involved (11, 12, 21, 28, 29; Sroka et al., submitted; Genco et al., unpublished). Depending on the strain and cultivation conditions, a variable amount of gingipains remain attached to the outer membrane or are secreted into the growth medium (16). While Kgp can function in hemoglobin binding, it may be premature to categorize it as an outer membrane receptor. The amino acid sequence of Kgp has no similarity to the TonB-dependent outer membrane proteins, indicating that a separate TonB-dependent outer membrane protein is responsible for binding and transport of heme from hemoglobin into the cell.

Previous studies in our laboratory have demonstrated that a common mechanism exists for the transport of both hemin and hemoglobin in *P. gingivalis*. In this study we report the characterization of the structural gene for a novel *P. gingivalis* TonB-dependent outer membrane receptor (HmuR) which is required for both hemoglobin and hemin binding and utilization in *P. gingivalis*. Inactivation of *hmuR* resulted in a diminished ability of *P. gingivalis* to bind hemoglobin and to grow with hemoglobin or hemin as sole iron sources. Furthermore, *E. coli* cells expressing the membrane-bound recombinant HmuR (rHmuR) were shown to bind both hemoglobin and hemin, and purified rHmuR was demonstrated to bind hemoglobin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *P. gingivalis* and *Escherichia coli* strains used in this study are indicated in Table 1. *P. gingivalis* wild-type strains were maintained on anaerobic blood agar (ABA) plates (Remel, Lenexa, Kans.). *P. gingivalis* strains WS1 was maintained on ABA plates supplemented with 1 μg of erythromycin per ml. All *P. gingivalis* cultures were incubated at 37°C in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) with 85% N_2, 5% H_2, and 10% CO_2 for 3 to 5 days. Following incubation at 37°C, cultures were inoculated in Anaerobe Broth MIC (Difco, Detroit, Mich.) and incubated at 37°C (under anaerobic conditions) for 24 h. *E. coli* was typically maintained in Luria-Bertani (LB) medium (Sigma, St. Louis, Mo.), supplemented with appropriate antibiotics and incubated aerobically with shaking.

To examine the ability of *P. gingivalis* to grow with different iron sources, *P. gingivalis* strains A7436 and WS1 were grown on anaerobic blood agar at 37°C for 3 days and then inoculated into Schaedler broth supplemented with 150 μM dipyridyl to chelate iron and incubated at 37°C under anaerobic conditions for 24 h. This served as the inoculum into Schaedler broth supplemented with 150 μM dipyridyl plus hemin (1.5 μM), hemoglobin (1.5 μM), or ferric chloride (100 μM). Prior to the addition of hemoglobin, 0.1% human serum albumin was added to sequester free heme. For some experiments, cultures were grown in basal medium (BM; Trypticase peptone, 10 g; tryptophan, 0.2 g; NaCl, 2.5 g; sodium sulfite, 0.1 g, and cysteine 0.4 g [per liter]) (13).

Isolation of the *P. gingivalis hmuR* locus. The *P. gingivalis hmuR* gene and upstream sequences were initially identified on a 5.3-kb DNA fragment from the A7436 cosmid library (36). The carboxy-terminal sequence was obtained by sequencing a second *P. gingivalis* strain A7436 clone which contained downstream DNA sequences. The *hmuR* DNA sequence was further confirmed by DNA sequence analysis of a PCR fragment corresponding to the entire *hmuR* gene. PCR amplification of *P. gingivalis* A7436 genomic DNA using primers F1 and R1 (Table 2) was carried out with *Vent* DNA polymerase (New England Biolabs, Beverly, Mass.) at 94°C for 1 min, 40°C for 2 min, and 72°C for 2 min for two cycles in a DNA Thermacycler 480 (Perkin-Elmer, Norwalk, Conn.). This was followed by 25 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min. The resulting PCR fragment was sequenced as described below. Southern blot analyses and genomic DNA isolations were performed as previously described (38).

DNA sequencing and computer analysis. DNA sequencing of *P. gingivalis* A7436 clones and the PCR fragment corresponding to the entire *hmuR* gene was performed using the PRISMTM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, Calif.) and 373A DNA sequencer. Computer analysis was performed as outlined by the Intelligenetics Suite and BLAST programs.

GenBank accession numbers. The sequences of the *hmuR* and *hmuY* genes were deposited into GenBank under accession numbers U87395 and 300705, respectively. The partial sequence of *hmuR* (previously designated *hemB*) was

TABLE 2. Primers and probes used in this study

Primer pair or probe[a]	Sequence	Description[b]
F1	ATAAGTTAAGAGGGAAATATG	Amplifies entire 1.94-kb *hmuR* gene
R1	CATTTCGCACCCATGCCGAAG	
F2	ACTGGAATTCGTGTAGTAACAAAGCAG	Amplifies 505 bp (8 to 493 nt) of *hmuR* gene; PCR product is probe 2
R2	ACTGAAGCTTTGATGATATTTGATAACACC	
F3	ACGTGAATTCGTGTAGTAACAAAGCAG	Amplifies 855 bp (8 to 853 nt) of *hmuR* gene
R3	GCTGATACGCCAGTTGGCA	
F4	GAAATGGATCAGGCTATCTAC	Amplifies 1.2-kb junction fragment of *hmuY-hmuR*
R4	GCTGATACGCCAGTTGGCA	
F5	GGTAAGCACCTGAAGACTTATG	Amplifies 469 bp (84 to 552 nt) of the *sod* gene
R5	CCAGTCAACAATACTCCAAAGA	
F6	GAAATGGATCAGGCTATCTAC	Amplifies 300 bp (85 to 384 nt) of the *hmuY* gene
R6	GAGTTCTCCATCCTGATA	
F7	ATGGCCAACCCTCCGGCCCAACCTA	Amplifies the *hmuR* gene without the signal peptide and without stop codon
R7	GAAAGTGATCCGAACCAACCCGTAT	
F8	ATGAAAAGTCTAGTAACAAAGCAGG	Amplifies the *hmuR* gene with signal peptide and without stop codon
R7	GAAAGTGATCCGAACCAACCCGTAT	
Probe 1		*Cla*I-*Cla*I-digested internal fragment (696 bp) of the *hmuR* (nt 791 to 1436) gene (has a *Hin*dIII site at nt 1387)

[a] F, forward; R, reverse.
[b] nt, nucleotide(s).

previously deposited under the same accession number and subsequently modified.

RT-PCR. *P. gingivalis* cultures were grown to the mid-logarithmic phase in anaerobic broth supplemented with 165 μM dipyridyl or anaerobic broth with dipyridyl plus hemin (1.5 μM). Total RNA was isolated using the RNagents Kit (Promega, Madison, Wis.). Samples were initially treated with DNase prior to reverse transcription-PCR (RT-PCR). To 1.0 μg of total RNA was added 1 μl of 10× DNase 1 buffer, 1 μl of DNase (Promega) and diethyl pyrocarbonate (DEPC)-treated water to achieve a final volume of 10 μl. Samples were incubated at room temperature for 15 min. DNase I was inactivated by the addition of 1 μl of 25 mM EDTA to the reaction mixture. The samples were then heated to 65°C for 10 min and placed on ice. Primers used in PCR included *hmuR*- and *sod*-specific primers, as well as a primer representing an *hmuY-hmuR*-specific junction fragment (Table 2). To the RNA samples was added 25 μl of 2× reaction mix, 100 ng of each primer, 1 μl of reverse transcriptase-*Taq* mix, and DEPC-treated water to a final volume of 50 μl. The samples were overlaid with mineral oil and placed in a DNA Thermocycler (Perkin-Elmer). cDNA synthesis was performed at 50°C for 30 min, followed by predenaturation at 94°C for 2 min. PCR amplification was carried out using the following parameters: denaturation at 94°C for 1 min, annealing at 54°C for 2 min, and elongation at 72°C for 2 min, for 30 cycles.

Construction and isolation of a *P. gingivalis hmuR* mutant. Primers F2 and R2 were used to amplify the region corresponding to bp 8 to 493 of the *hmuR* gene, yielding a DNA fragment of 485 bp (Table 2). To the forward primer, four nonspecific bases and an *Eco*RI restriction site were added. To the reverse primer, four nonspecific bases and an *Hin*dIII site were added. These additions increased the final size of the PCR product to 505 bp. This PCR fragment was cloned into pGEM3z (Promega), and the *hmuR* fragment was then interrupted by the insertion of the *ermF* gene (32) into the *Pst*I site of the *hmuR* DNA fragment. The resulting plasmid (pWS1) was transformed into *E. coli* JM109 (Promega), and the insertion of *ermF* (with flanking sequences) (31) into the *hmuR* fragment was confirmed by DNA sequencing. pWS1 was introduced into *P. gingivalis* A7436 by electroporation briefly as follows. *P. gingivalis* A7436 was inoculated into anaerobe broth to an initial optical density at 660 nm (OD_{660}) of 0.1 and incubated anaerobically for 6 h (final OD_{660} = 0.4). The *P. gingivalis* culture was then centrifuged at 10,000 × *g* for 10 min and washed with electroporation buffer (1 mM $MgCl_2$, 10% glycerol), and the pellet was mixed with 200 ng of pWS1 DNA and placed in a 2.5-cm electroporation cuvette. Electroporation was carried out at 25 μF, 200 Ω, and 2.5 V and resulted in time constants of 3.1 to 3.4 s. The *P. gingivalis* A7436 alone was also electroporated and used as a negative control. After electroporation, 800 μl of the anaerobic broth was added, and the cells were incubated overnight at 37°C under anaerobic conditions. Samples were centrifuged, 900 μl of supernatant was removed, the pellet was resuspended in the remaining 100 μl of supernatant, and the culture was plated onto an ABA plate containing 1 μg of erythromycin per ml. The plates were incubated under anaerobic conditions at 37°C for 7 to 10 days as described above. Individual transformants were isolated, and insertion of the *ermF* gene in the *P. gingivalis hmuR* mutants (WS1, WS2, WS4, and WS5) was confirmed by Southern blot analysis. The mutation in the *hmuR* gene was further confirmed in *P. gingivalis* WS1 by PCR analysis using primers specific for the 5′ and 3′ portions of the *hmuR* gene.

Construction of the HmuR expression plasmid. The *hmuR* gene was PCR amplified from 100 ng of total genomic DNA obtained from *P. gingivalis* A7436 (94°C for 30 s, 60°C for 30 s, and 72°C for 2 min, followed by 30 min at 72°C; 25 cycles). The forward primers (F6 and F7, Table 2) were designed to produce *hmuR* either with or without its native signal peptide sequence. The reverse primer (R6, Table 2) was designed to remove the native stop codon and preserve the reading frame through the C-terminal tag. The amplified products were purified and cloned into the vector pCRT7/CT-TOPO (Invitrogen, Carlsbad, Calif.), which contains sequences coding for the V5 epitope and polyhistidine (His_6) regions. The resulting plasmids (pTO1 and pTO2) were transformed into *E. coli* TOP10F′, and transformants were selected on LB plates containing 100 μg of ampicillin per ml. The *hmuR* insert was confirmed by restriction analysis, PCR, and DNA sequence analysis.

Expression of rHmuR in *E. coli*. *E. coli* BL21(DE3)pLysE cells (Invitrogen) were transformed with pTO1 or pTO2, and transformants were selected on LB medium or minimal medium (M9) containing 100 μg of ampicillin and 34 μg of chloramphenicol per ml. Then, 1 ml of the overnight culture was inoculated into fresh 10 ml of LB medium or M9 supplemented with both antibiotics and grown at 37°C to an OD_{600} of 0.5 to 0.6. To induce the expression of the cloned *P. gingivalis hmuR* gene, isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) was added to a final concentration of 0.5 to 1.0 mM, and growth was continued for 5 h. Samples were removed at hourly intervals, centrifuged, and frozen at −20°C. *E. coli* cells harboring a plasmid expressing the *lacZ* gene, pCRT7/CT-LacZ (Invitrogen, Carlsbad, Calif.) was utilized as a positive control, and the vector alone was used as a negative control.

SDS-PAGE and Western blotting. Samples taken before and 1 to 5 h after IPTG induction were suspended in 2 × Laemmli sample buffer, boiled for 5 min and examined by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) on 12% gels (22). The proteins were either stained with Coomassie brilliant blue R-250 (CBB; Bio-Rad, Hercules, Calif.) or were transferred (43) onto nitrocellulose membranes (Bio-Rad) in 30 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (pH 11.0; Sigma) for 1 h at constant current of 0.2 A. Western blotting was carried out according to the

method of Burnette (7) with slight modifications. Membranes were incubated for 2 h at room temperature in 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl (TBS) and 3% skim milk. After washing with TBS containing 0.05% Tween 20 (TTBS), anti-fusion protein antibody conjugated with horseradish peroxidase (mouse anti-V5-HRP; Invitrogen) in TBS containing 1% skim milk was added, and this was incubated for 2 h at room temperature. Membranes were washed with TTBS and in the final step with TBS. Chemiluminescence detection was performed within 1 min at room temperature using the ECL System (Amersham Pharmacia Biotech, Piscataway, N.J.). Autoradiography films (Amersham Pharmacia Biotech) were exposed for 1 to 5 min and then developed. Electrophoresis of rHmuR purified from membrane fraction, for N-terminal sequencing, was carried out according to the method of Schagger and von Jagow (35) and transferred onto a polyvinylidene difluoride (PVDF) (Bio-Rad) membrane as indicated above.

Purification of rHmuR. Following a 5-h IPTG induction period *E. coli* BL21(DE3)pLysE cells harboring pTO1 or pTO2 were harvested by centrifugation for 20 min at 8,000 × *g*. The pellet was resuspended in 20 mM phosphate buffer (pH 7.4) containing 0.14 M NaCl (PBS), supplemented with protease inhibitors (Complete EDTA-free; Roche Molecular Biochemicals, Indianapolis, Ind.), frozen and thawed three times, and passed through a French press. After centrifugation for 15 min at 25,000 × *g*, the pellet (containing inclusion bodies) was resuspended in PBS containing protease inhibitors. The remaining supernatant was centrifuged for 1 h at 70,000 × *g* to obtain the total membrane fraction. To purify rHmuR, frozen samples containing inclusion bodies or samples containing membrane fractions were thawed, and purification was performed according to Invitrogen's procedure using Ni-chelate chromatography under denaturing conditions. The protein was eluted from the column with urea buffer (pH 4.0), dialyzed against PBS containing decreasing concentrations of urea and 0.1% octyl-D-glucopyranoside (OG; Sigma) and finally dialyzed against PBS containing 0.5 M urea and 0.1% OG. After centrifugation samples were concentrated using Centriprep-10 (Amicon, Beverly, Mass.), and the protein concentration was determined by the bicinchoninic acid method (39).

Hemoglobin binding to rHmuR. rHmuR purified using Ni-chelate chromatography was transferred onto a nitrocellulose membrane and probed with 100 ng of human hemoglobin (Sigma) per ml, which was biotinylated (18) according to the Pierce's protocol (Pierce, Rockford, Ill.). Hemoglobin binding to rHmuR was determined using streptavidin conjugated with horseradish peroxidase (Roche Molecular Biochemicals) and chemiluminescence detection as described above.

Binding of hemoglobin and hemin by *E. coli* cells expressing HmuR. Detection of rHmuR on the surface on *E. coli* strain BL21(DE3)pLysE was carried out by dot blot assay using antibodies to the fusion protein as discussed above. *E. coli* expressing rHmuR deposited in inclusion bodies (cells transformed with pTO1) or membrane bound (cells transformed with pTO2), and cells containing plasmid alone were harvested before and after IPTG induction, washed with PBS, and adjusted to an OD_{600} of 1.0. Aliquots of the cell suspension (0.8 ml) were mixed with 0.2 ml of human hemoglobin dissolved in PBS (final concentration, 5 μM) or hemin dissolved in dimethyl sulfoxide (final concentration, 10 μM). Samples were incubated for 1 h at 37°C and centrifuged, and the OD_{400} of the resulting supernatant was determined. Adsorbed hemoglobin or hemin was evaluated by determining the decrease of the absorbance of the supernatant and was recorded as the percentage of the initial hemoglobin or hemin. Samples containing hemoglobin, hemin, or cells only were incubated under the same conditions and served as appropriate controls.

RESULTS

Characterization of the *P. gingivalis hmuR* gene. To identify genes required for iron transport from hemin and hemoglobin in *P. gingivalis*, we initially utilized transpositional mutagenesis with the *Bacteroides fragilis* transposon Tn*4351* and identified a mutant of *P. gingivalis* (MSM-3) which grew poorly with hemin or hemoglobin as sole iron sources (14). Further characterization of *P. gingivalis* MSM-3 revealed that introduction of Tn*4351* resulted in the mobilization of the endogenous insertion sequence element IS*1126* in the *P. gingivalis* MSM-3 genome (36). Characterization of the first additional IS*1126* insertion site revealed that it had inserted into the promoter region of the gene encoding the *P. gingivalis* Kgp protein (*kgp*). The hemin-hemoglobin defect in *P. gingivalis* MSM-3 was thus attributed to the inactivation of *kgp* (36). To characterize the second additional IS1126 insertion site, an oligonucleotide specific to its flanking sequences was used to probe a *P. gingivalis* A7436 cosmid library. Nucleotide sequencing of a positive clone resulted in the fortuitous identification of a novel *P. gingivalis* gene (*hmuR*), which is characterized in this study. The initial 1,050 bp of the *P. gingivalis hmuR* gene was identified as part of a 5.3-kb DNA fragment from the *P. gingivalis* A7436 cosmid library. The DNA sequence corresponding to the carboxy terminus of *hmuR* was obtained following sequencing of a second clone containing downstream sequences. The sequence of the entire *hmuR* gene from strain A7436 was further confirmed following sequencing of a PCR fragment obtained from strain A7436 using primers F1 and R1 (see Table 2). The *hmuR* gene from strain A7436 is composed of 1,941 nucleotides and encodes for a 73-kDa predicted protein with a pI of 8.8. Analysis of the HmuR predicted protein using the SignalP program revealed a likely signal peptide cleavage site between Ala^{24} and Ala^{25}. Further analysis using the Kyte and Doolittle plot program demonstrated that HmuR is hydrophobic, as is typical of outer membrane receptors (data not shown).

The *P. gingivalis hmuR* gene shares homology with genes whose products have been shown to be TonB-dependent outer membrane receptors involved in iron acquisition. These include the *Y. enterocolitica* HemR (55% identity), which is a member of a well-defined hemin uptake operon, the *Shigella dysenteriae* ShuA (54% identity); the *E. coli* CirA, FhuE, and ChuA (42, 39, and 51% identities, respectively); the *Campylobacter coli* CfrA (41% identity); and the *V. cholerae* IrgA (39% identity). Two regions of the translated open reading frame (ORF) of HmuR (residues 33 to 39 and 135 to 170) exhibited extensive sequence similarity to TonB boxes I and IV; homology between the *P. gingivalis hmuR* gene and the TonB-dependent receptors was most pronounced in the region which corresponds to TonB IV (Fig. 1A).

As we were conducting these studies, a gene from *P. gingivalis* 53977 (*hemR*), which also exhibits homology to genes involved in iron acquisition from several gram-negative organisms, was identified (19). Comparison of the *hmuR* and *hemR* sequences revealed that the N-terminal region of the *hmuR* gene was identical to the initial 516 bases of the *P. gingivalis hemR* gene. However, after bp 516, no identity was observed between the *P. gingivalis hmuR* and *hemR* genes (36). HemR exhibits homology to *Vibrio cholerae* IrgA (41%), *Y. enterocolitica* HemR (25%), *E. coli* BtuB (36%), *E. coli* CirA (35%), *E. coli* IutA (29%), *E. coli* FecA (29%), *E. coli* FhuA (25%), and *Y. enterocolitica* FoxA (27%). Interestingly, we found that the carboxy-terminal region of HmuR exhibited significant sequence similarity to proteins involved in heme and hemoglobin binding and utilization (Fig. 1B). These include the major hemoglobin receptors in *N. gonorrhoeae* and *N. meningitidis*, HmbR and HpuB (35 and 41% identity, respectively), and the hemoglobin receptors in *H. ducreyi* HgbA (48% identity) and *H. influenzae* HgpB (41% identity) (Fig. 1B and references 8, 25, 26, 34, and 40). Amino acid comparisons of the conserved domains of these heme and hemoglobin receptors, as well as several siderophore and vitamin B_{12} receptors, revealed a highly conserved receptor domain containing invariant histidine residues and FRAP and NPNL amino acid boxes (6). These residues were also conserved in the *P. gingivalis* HmuR hemoglobin receptor (Fig. 1B). The conserved histidine residues were present in the *P. gingivalis* HmuR protein at positions 95 and 434, an Arg-Ala-Pro sequence from residues 421 to 423, and an Asp-Pro-Asp-Leu motif from residues 442 to 445. We also identified a number of conserved glutamic acid residues which were common to *P. gingivalis* HmuR and to several of the heme and hemoglobin receptors (Fig. 1B).

Upstream of the *hmuR* gene we identified an ORF of 429 bp predicted to encode a 143-amino-acid (aa) protein which we designated *hmuY*. Sequence analysis of HmuY revealed an ATP-GTP-binding loop (aa 21 to 28), suggesting that it may function as an ATPase. The *hmuY* gene exhibited 99% identity

A **Ton B box IV**

Heme, Hb, Hb-Hp receptors

Consensus		**PDLIERIEVIRGPSSALYGS-ALGGVVNIITKKAQQ**	
Pg HmuR	135	PDDIERIEVLRGASSALYGSNAIGGVINIITRTAKD	170
Pg HemR	135	PDDIERIEVLRGASSALYGSNAIGGVINIITRTAKD	170
Vv HupA	127	PDMLKSVEIVKGAASSLHGSDAIGGVVAFETKDPAD	163
Vc HutA	136	TDMVKSVEIVKGAASSLQGSDAIGGIVAFETKDPAD	162
Pa PhuR	130	PDIVKRVEILRGPASALYGSNAIGGAVSYFTLDPSD	165
Ye HemR	136	PALVKRVEIVRGPSALLYGSGALGGVISYETVDAAD	171
Yp HmuR	136	PALVKRIEIVRGPAALLYGSGALGGVIAYETVDAAD	171
Sd ShuA	122	PALIKRVEIVRGPSALLYGSGALGGVISYDTVDAKD	157
Hi HgpA	185	IETLKQVTIRKGADSLKSGSGALGGSVSLDTKDARD	220
Hi HgpB	173	IETLKEVNITKGADSIKNGSGSLGGSVIYKTKDARD	209
Nm HpuB	135	PENFSEVTITKGADSLKSGSGALGGAVNYQTKSASD	160
Hi HhuA	137	IETLKQVTIRKGADSLKSGSGALGGSVSFDTKDARD	171
Pf PfuR	126	PEIVKRVEILRGPASALYGSSAIGGAVSYFTLDPQD	162

Siderophore receptors

Ec R4	102	--AIERIEVIRGPMSTLYGSDAMGGVVNIITRKNAD	136
Ec FepA	138	PEMIERIEVLRGPAAARYGNGAAGGVVNIITKKGSG	173
Pa PfeA	143	ADQVERIEVIRGPAAARYGNGAAGGVVNIITKQAGA	178
Bp BfeA	147	AEEVERIEVIRGPAAARYGSGAMGGVVNIITKRPAD	182
Cc CfrA	126	ISSIERIEVIKGPMSTLYGSEALGGVVNIITKKVSD	162

Vitamin B_{12} receptors

Ec BtuB	121	IALVQRVEYIRGPRSAVYGSDAIGGVVNII--TTRD	155
St BtuB	121	VSLVQRIEYIRGPPSAIYGSDAIGGVVNII--TTRD	155

B **Conserved amino acid motifs and amino acid residues**

Heme, Hb, Hb-Hp receptors

Pg HmuR	418	EG**Y**R**AP**SLQEMYFFFNHGAFFIYG**NPDL**KPEKSRMLSYSAE	458
Vv HupA	471	QG**FRAP**SFNELYYTYDNPGHGYTNR**PNPNL**ESEKSLSY**E**	508
Vc HutA	458	QG**FRAP**DFQELYYSFGNPA**H**GYVFK**PNPNL**EAEDSVSY**E**	497
Sd ShuA	432	QA**FRAP**TMGEMYNDSK**H**FSIGRFYTNYWVP**NPNL**RPETNETQE	474
Ye HemR	445	QA**FRAP**TMGEMYNDSK**H**FSMNIMGNTLTNYWV**PNPNL**KPETNETQE	490
Hi HgpA	811	KG**FRAP**TSDEIYFTFK**H**PDFSIQ**PNRDL**QPETAKTKE	847
Hd HgbA	690	TG**FRAP**TSDEIYFVFQ**H**PSFSIY**PNLYL**KAERSKNKE	726
Hd HupA	690	TG**FRAP**TSDEIYFVFQ**H**PSFSIY**PNLYL**KAERSKNKE	723
Hi HgpB	730	KG**FRTP**TSDEMYFTFK**H**PDFTILP**NADL**KPEIAKTKE	766
Nm HpuB	533	TG**FRAP**TSDETWLLFP**H**PDFYLKA**NPNL**KAEKAKNWE	567
Hi HhuA	759	KA**FRAP**TSDEIYFTFL**H**PDFSIRP**NRDL**QAETAKTKE	795
Pf PfhR	504	EG**FRTP**SAKALYGRFENLNLGYTVEP**NPDL**KPETSKGIE	542
Pa PhuR	508	QG**FRTP**TAKALYGRFENLQAGYHIEP**NPNL**KPEKSQSFE	546

Siderophore receptors

Ec R4	412	TG**Y**K**AP**RMGQLHKGISGVSGQGKTNLLG**NPNL**KPEESVSY**E**	452
Ec FepA	486	RA**Y**K**AP**SLYQTNPNYILYSKGQGCYASAGGCYLQ**GNDDL**KAETSINK**E**	533
Pa PfeA	486	RA**Y**K**AP**NLYQLNPDYLLYSRGQGCYGQSTSCYLR**GNDGL**KAETSVNK**E**	533
Bp BfeA	483	RA**Y**K**AP**NLYQSNPNYLLYSRGNGCLASQTNTNGCYLV**GNEDL**SPETSVNK**E**	533
Cc CfrA	446	TG**FRTP**YANRLINGTYSYSSGQGRFPTY**GNPDL**KEETSLNY**E**	486

Vitamin B_{12} receptors

Ec BtuB	411	TS**Y**K**AP**NLGQLYGFYG**NPNL**DP**E**KSKQW**E**GAFEG	444
St BtuB	411	TS**Y**K**AP**NLGQLYGYYG**NPNL**NP**E**KSKQW**E**GAFEG	444

FIG. 1. Conserved TonB Box IV, amino acid motifs, and amino acid residues in the *P. gingivalis* HmuR protein. (A) Homology between the *P. gingivalis* HmuR and the TonB box IV regions of several different heme and hemoglobin receptors and siderophore and vitamin B_{12} receptors. The *E. coli* TonB consensus sequence is also depicted. (B) Homology between the *P. gingivalis* HmuR protein and the carboxy-terminal region of several different heme and hemoglobin receptors and siderophore and vitamin B_{12} receptors. Conserved amino acids between the *P. gingivalis* HmuR and the consensus sequence are indicated by boldface letters. The numbers indicate the position in the unprocessed protein of the first amino acid listed. Pg, *P. gingivalis*; Vv, *V. vulnificus*; Vc, *V. cholerae*; Sd, *S. dysenteriae*; Ye, *Y. enterocolitica*; Hi, *H. influenzae*; Hd, *H. ducreyi*; Nm, *N. meningitidis*; Pf, *P. fluorenscens*; and Pa, *P. aeruginosa*.

with a previously identified ORF (ORF1) located upstream of the *P. gingivalis hemR* gene in strain 53977 which has been proposed to function as a DNA binding protein (19). Located 228 bp upstream of the *hmuY* start codon, a 19-bp putative Fur box was identified (5′-GATAATTATGAAAAAAATC-3′; see Fig. 4). This Fur box is identical to that found upstream of ORF1 (18) and exhibits 68% identity (13 of 19 bases identical) to the *E. coli* consensus Fur box sequence. Internal regions of HmuY exhibited 76 and 89% identities with two peptides previously demonstrated to bind hemin as assessed by SDS-PAGE and TMBZ analysis (20). Located 36 bp downstream of *hmuR* in *P. gingivalis* A7436, we identified an ORF which shares homology with the gene encoding Mg chelatase (*mg che*). Interestingly, we found that in strain A7436 this gene was disrupted by an insertion sequence exhibiting 100% identity to the *E. coli* IS*10* element (see Fig. 4).

Presence of *hmuR* in different *P. gingivalis* strains. To confirm the presence of a single copy of *hmuR* in *P. gingivalis*, a probe derived from the carboxyl terminus (probe 1, Table 2) which is specific for *hmuR* was used in Southern blot analysis. Digestion of DNA derived from *P. gingivalis* A7436 with various restriction enzymes confirmed that *hmuR* is present in a single copy in this strain (Fig. 2A). A search of the unfinished *P. gingivalis* strain W83 genomic sequence database of The

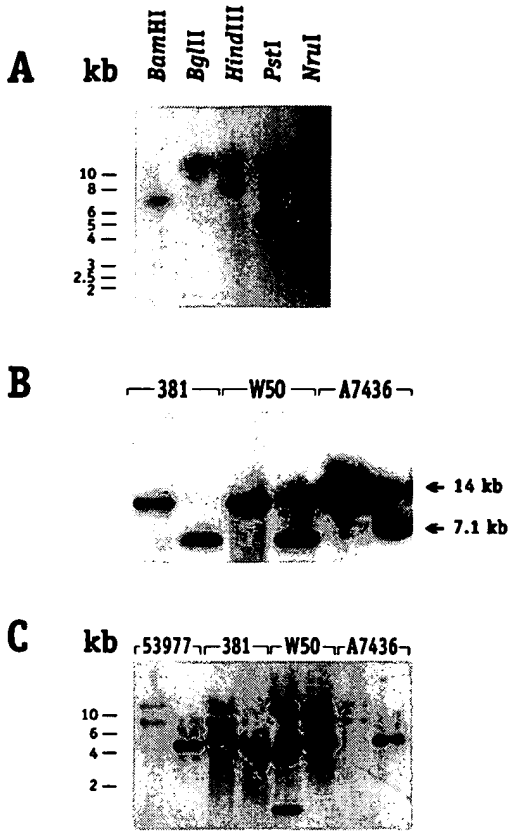

FIG. 2. Southern blot analysis of DNA from *P. gingivalis* strains using an *hmuR*-specific probe. (A) *P. gingivalis* A7436 genomic DNA digested with various enzymes as indicated. The probe used was a carboxy-terminal probe (probe 1, Table 2). (B) Southern blot analysis of chromosomal DNA from *P. gingivalis* strains 381, W50, and A7436 digested with *Hin*dIII (first lane for each strain) and *Pst*I (second lane for each strain). The probe used was an amino terminal probe (probe 2, Table 2). Fragment sizes are indicated with arrows. (C) Southern blot analysis of chromosomal DNA from *P. gingivalis* strains 53977, 381, W50, and A7436 digested with *Hin*dIII (first lane for each strain) and *Pst*I (second lane for each strain). The probe used in both panels A and C was a carboxy-terminal *Cla*I-*Cla*I fragment of *hmuR* which contains an *Hin*dIII site at nucleotide 1387 (probe 1, Table 2).

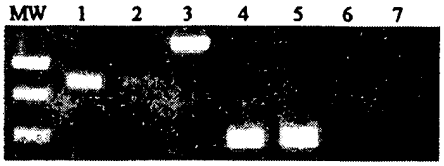

FIG. 3. RT-PCR analysis of *hmuY* and *hmuR* transcription. Total RNA was extracted from *P. gingivalis* grown under iron-replete (lanes 2, 4, and 6) and iron-depleted (lanes 1, 3, 5, and 7) conditions. RT-PCR was performed using the primers indicated in Table 2. Lane M, molecular weight standards; lanes 1 and 2, *hmuR*; lane 3, *hmuY-hmuR* junction; lanes 4 and 5, *sod*; lanes 6 and 7, *Taq* negative control using primers to amplify 300 bp of *hmuY* (F6 and R6, Table 2).

Institute for Genome Research (TIGR [http://www.tigr.org]), also revealed the presence of an ORF that exhibited 99% homology to the *hmuR* gene from strain A7436 (data not shown). To further confirm that *hmuR* was present in other *P. gingivalis* strains, Southern blot analysis with an N-terminal probe (probe 2, Table 2) was performed as shown in Fig. 2B. We observed a similar banding pattern in the *P. gingivalis* strains examined (A7436, W50, and 381), indicating that the N-terminal region of the *hmuR* gene is highly conserved. Since the probe used also recognizes a sequence present within the *hemR* gene, we cannot, however, rule out the possibility that observed reactivity is due to *hemR* sequences, which may exist in strains W50 and 381.

The lack of homology between the 3′ ends of *hemR* gene from strain 53977 and *hmuR* gene from strain A7436 led us to speculate that genomic variation may exist within the carboxy termini of *hmuR* genes of different *P. gingivalis* strains. To assess the genomic variability in the *hmuR* gene and to determine if *hmuR* was present in strain 53977, probe 1 (Table 2) was utilized in Southern blot analysis with DNA from strains 53977, 381, W50, and A7436. As shown in Fig. 2C, we observed variability in bands corresponding to the carboxy terminus of *hmuR* in the *P. gingivalis* strains examined. The probe derived from this portion of the gene hybridized to 8.0- and 13.5-kb *Hin*dIII and 4.0-kb *Pst*I fragments in strains A7436 and 53977 DNA, 7.5- and 10.0-kb *Hin*dIII and 3.6-kb *Pst*I fragments in strain 381 DNA, and 4.5- and 7.5-kb *Hin*dIII and 4.0-kb *Pst*I fragments in W50 DNA. These results indicate that there is a genetic variability in the carboxy terminus of *hmuR* among these *P. gingivalis* strains. Our results also suggest that additional variability may exist outside of the *hmuR* gene.

Transcription of *hmuR* and *hmuY* in response to iron limitation. The promoter region of *hmuY* contains a putative Fur consensus binding sequence (13 of 19 bases identical to the *E. coli* Fur consensus box) which could serve to regulate the expression of both the *hmuY* and the *hmuR* genes. This is further supported by the absence of −10 and −35 promoter sequences upstream from the putative transcriptional start site of the *P. gingivalis hmuR* gene. To examine the regulation of *hmuY* and *hmuR* genes, RT-PCR analysis was performed with RNA preparations from *P. gingivalis* grown in iron-depleted and iron-replete conditions. Prior to conducting the RT-PCR experiment, all primers were used in standard PCR reactions to test for functionality and to determine the proper annealing and extension conditions. *P. gingivalis* was passaged without iron or hemin in anaerobic broth with an iron chelator (165 μM dipyridyl), and this served as the inoculum into anaerobic broth with dipyridyl and anaerobe broth containing dipyridyl and hemin. RNA was isolated from these cultures, and primers specific to the initial 845 bp of the *hmuR* gene (F3 and R3, see Table 2) and 469 bp of the *P. gingivalis sod* gene (F5 and R5, Table 2) were used in RT-PCR analysis (Fig. 3). We found that under iron depletion an *hmuR* transcript was synthesized and that the level of the *hmuR* transcript appeared to be greater than that observed in organisms grown without added iron but with added hemin. The increased transcription of *hmuR* does not appear to be due to growth-dependent expression, since the level of the *sod* transcript was similar under iron-depleted and heme-replete conditions. This finding correlates with a recent study in which Lynch and Kuramitsu (26) demonstrated that the transcription of the *P. gingivalis sod* gene was dependent on growth but was not affected by iron depletion. Our studies also demonstrated repression of the *hmuR* transcript when *P. gingivalis* A7436 was grown with 100 μM ferric chloride (data not shown).

To determine if *hmuY* and *hmuR* were cotranscribed, we used primers which would amplify an *hmuY-hmuR*-specific junction fragment (F4 and R4, Table 2) in RT-PCR with RNA obtained from *P. gingivalis* A7436 grown in anaerobic broth with dipyridyl. A fragment representing the *hmuY-hmuR* specific junction transcript was amplified using these primers (Fig. 3), indicating that both genes are cotranscribed. Taken together, these results indicate that the *hmuY* and *hmuR* genes

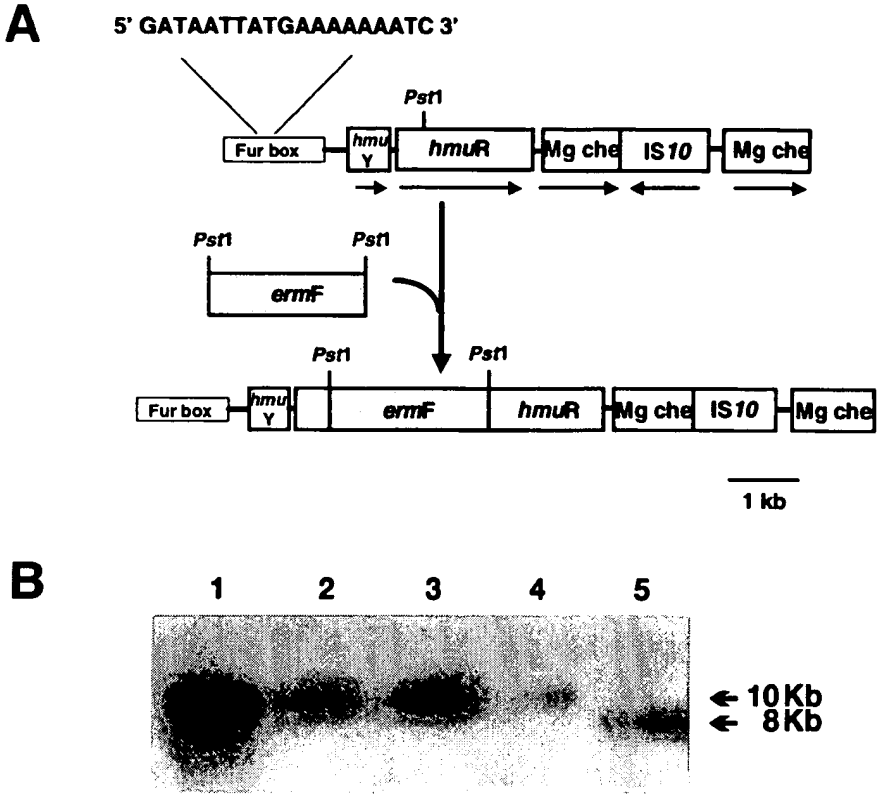

FIG. 4. Construction of the *hmuR* mutant WS1. (A) The *P. gingivalis hmuR* insertional mutant (WS1) was constructed following insertion of the *ermF* cassette (with flanking sequences) in the *Pst*I site of *hmuR*. The direction of transcription is indicated by an arrow, and the *Pst*I restriction site(s) of *hmuR* and *ermF* are noted. Also indicated is the map of *hmuR* region from *P. gingivalis* A7436. Upstream of the *hmuR* gene we identified an ORF of 438 bp (*hmuY*), predicted to encode a 145-aa protein. The promoter region of *hmuY* contains a putative Fur consensus binding sequence (13 of 19 bases identical to the *E. coli* Fur box; the Fur box is not drawn to scale). Located downstream of *hmuR*, an ORF which encodes a putative Mg chelatase (*mg che*), which was disrupted by a gene encoding a IS*10*-like element, was identified. (B) Southern blot analysis of chromosomal DNA from *P. gingivalis hmuR* mutants probed with an *hmuR*-specific probe (see Table 2). Lanes 1 to 4, genomic DNA from four separate transformants (WS1, WS2, WS4, and WS5); lane 5, genomic DNA from A7436. Introduction of the *ermF* cassette adds ~2.0 kb, causing a shift in the *hmuR* band.

are cotranscribed and suggest that transcription is increased under iron-limiting conditions.

Characterization of a *P. gingivalis hmuR* mutant. Based on results obtained from the amino acid sequence analysis of HmuR, we postulated that HmuR could function as an iron-regulated TonB-dependent outer membrane receptor for the acquisition of iron from hemin and/or hemoglobin in *P. gingivalis*. To define the function of the *hmuR* gene in *P. gingivalis*, we constructed a *P. gingivalis hmuR* mutant by insertional inactivation using the *ermF* cassette (Fig. 4) and confirmed the insertion of the *ermF* cassette by Southern blot analysis. We observed an ~2-kb shift in the DNA band corresponding to the *hmuR* gene in four separately isolated *P. gingivalis* transformants (Fig. 4B). *P. gingivalis* strain WS1 was chosen for further analysis, and the insertion of the *ermF* cassette in the *hmuR* gene was further confirmed by PCR analysis using 5′ and 3′ *hmuR*-specific primers (data not shown).

The ability of *P. gingivalis* WS1 to grow with hemin and hemoglobin as sole sources of iron was then examined. *P. gingivalis* cultures were grown for 24 h in Schaedler broth medium with 150 μM dipyridyl to chelate iron, and this served as the inoculum into Schaedler broth plus dipyridyl or Schaedler broth plus dipyridyl supplemented with hemin (1.5 μM), hemoglobin (1.5 μM), or ferric chloride (100 μM). Growth of *P. gingivalis* strain A7436 in Schaedler broth plus dipyridyl supplemented with hemin, hemoglobin, or ferric chloride resulted in a typical growth curve with final OD_{660} values of 0.71, 0.59, and 1.1, respectively, after 63 h of growth (Fig. 5). In contrast, *P. gingivalis* WS1 exhibited diminished growth with either hemin or hemoglobin, with final OD_{660} values of 0.19 and 0.23, respectively (Fig. 5). The poor growth of *P. gingivalis* WS1 with hemin or hemoglobin does not appear to result from a generalized growth defect since this strain grew similarly to *P. gingivalis* A7436 in Schaedler broth plus dipyridyl supplemented with 100 μM ferric chloride (final OD_{660} of 0.91). This finding indicates that HmuR is specific for the uptake of heme-containing compounds such as hemin and hemoglobin, but the uptake of inorganic iron (ferric chloride) is mediated by another mechanism. In addition, these results indicate that hemin and hemoglobin utilization in *P. gingivalis* occur through a common HmuR-mediated mechanism. We also found that hemoglobin was an effective competitor for the transport of radiolabeled hemin in *P. gingivalis* A7436 (data not shown), further supporting a common mechanism for hemin and hemoglobin utilization in *P. gingivalis*.

Disruption of *hmuR* correlates with diminished hemoglobin binding. To determine if the inability of *P. gingivalis* WS1 to grow with hemoglobin was due to a decreased ability to bind hemoglobin, we examined the binding of *P. gingivalis* whole cells to hemoglobin by using a spectrophotometric assay (29).

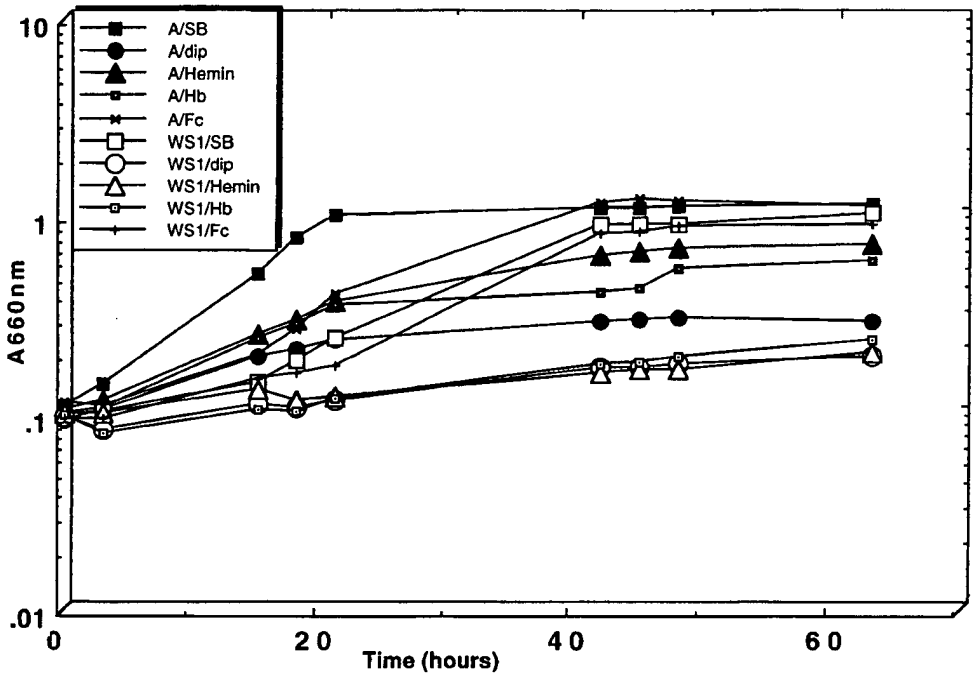

FIG. 5. Growth of *P. gingivalis* A7436 (A) and WS1 with hemin, hemoglobin, and ferric chloride. Cultures were initially starved in Schaedler broth supplemented with 150 μM dipyridyl for 24 h. This was used to inoculate Schaedler broth alone (SB), Schaedler broth plus 150 μM dipyridyl (dip), Schaedler broth plus 150 μM dipyridyl plus 1.5 μM hemin (Hemin), Schaedler broth plus 150 μM dipyridyl plus 1.5 μM hemoglobin (Hb), or Schaedler broth plus 150 μM dipyridyl plus 100 μM ferric chloride (Fc). The results are representative of two experiments.

P. gingivalis cells were grown anaerobically overnight in BM. The percent absorbance was calculated relative to the control strain A7436, which was set at 100%. *P. gingivalis* WS1 exhibited a significant decrease in hemoglobin binding compared to the parent strain A7436. *P. gingivalis* WS1 bound 34% less hemoglobin than did the parental strain A7436 (data not shown). The observation that the *hmuR* mutant did not exhibit a total decrease in hemoglobin binding may be due to the presence of multiple hemoglobin binding proteins in *P. gingivalis*, including Kgp and HRgpA (12, 21, 24, 28, 29), as has been described for other gram-negative organisms (23, 27, 40). This idea was supported by the observation that the *P. gingivalis* Kgp mutant (strain MSM-3) also bound less hemoglobin than the wild-type strain A7436 (data not shown).

Expression of rHmuR and characterization of hemin and hemoglobin binding. To further confirm the ability of HmuR to bind hemoglobin, we overexpressed the protein in *E. coli* and examined hemoglobin binding by recombinant strains. Plasmids containing *hmuR* either with (pTO2) or without (pTO1) its native signal peptide were subsequently transformed into *E. coli*. The HmuR expression level of the resulting *E. coli* BL21(DE3)pLysE strain harboring pTO1 was monitored by SDS-PAGE (Fig. 6A) and after transfer onto nitrocellulose membrane by detection with antibody against the fusion protein (Fig. 6B). Basal level expression of rHmuR was exhibited prior to the addition of IPTG in *E. coli* grown in LB medium (data not shown), as well as in M9 medium (Fig. 6B); however, an increase in the expression of the protein after IPTG induction was exhibited in *E. coli* grown in M9 medium. We did not detect new protein bands following IPTG induction in bacteria transformed with the vector alone (Fig. 6A), and no protein bands were visible on the immunoblot after probing with the anti-fusion protein antibody (Fig. 6B). Following IPTG induction, the expressed rHmuR together with the fusion tag attached to the C terminus of the protein possessed a molecular mass of approximately 80 kDa. We also observed several additional protein bands which may correspond to degradation products of rHmuR (Fig. 6A). This was further confirmed by Western blot analysis using antibodies to the fusion protein (Fig. 6B). The ability of the purified HmuR protein to bind hemoglobin was next examined by a solid-phase assay. As shown in Fig. 6C, rHmuR isolated from inclusion bodies bound human hemoglobin.

We next expressed HmuR containing its native signal peptide to export and localize this protein in outer membranes of *E. coli* cells. SDS-PAGE and Western blot analysis showed that rHmuR was associated with the membrane fraction (Fig. 7A and B). As shown in Fig. 7C, rHmuR was expressed on the surface of *E. coli* BL21(DE3)pLysE strain harboring pTO2 as detected by antibodies to the fusion protein. Low basal level expression of rHmuR was exhibited prior to the addition of IPTG in *E. coli* grown in LB (data not shown), as well as in M9 medium (Fig. 7B). The membrane bound rHmuR expression level of the resulting *E. coli* harboring pTO2 was lower compared with rHmuR deposited in inclusion bodies in *E. coli* transformed with pTO1 (Fig. 7A). This result was expected, as the addition of the C-terminal His tag blocked the C-terminal Phe residue, which has been shown to be highly conserved and necessary for the stable incorporation of a protein into the outer membrane. Following IPTG induction the expressed rHmuR, together with the fusion tag attached to the C terminus of the protein, possessed a molecular mass of approximately 80 kDa. We also observed several additional protein bands which may correspond to degradation products of rHmuR (Fig. 7A), and this was further confirmed by Western blot analysis using the anti-fusion protein antibody (Fig. 7B). The

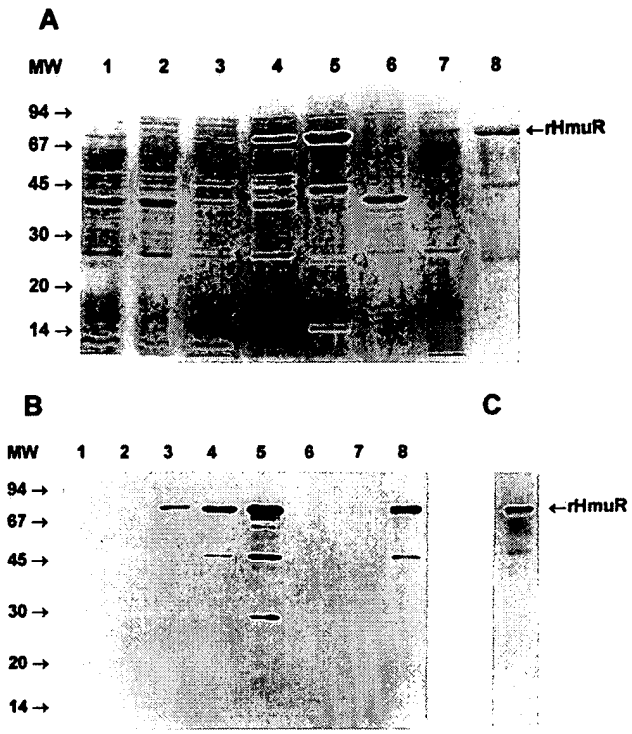

FIG. 6. Expression, purification, and hemoglobin binding activity of rHmuR localized in inclusion bodies. (A) Expression of rHmuR. The gene encoding the protein lacking the signal peptide was cloned into pCRT7/CT-TOPO and expressed in *E. coli* BL21(DE3)pLysE. CBB-stained SDS-PAGE gel of cells harboring the vector alone (lane 1, uninduced; lane 2, induced), cells expressing rHmuR (lane 3, uninduced; lane 4, induced), inclusion body fraction (lane 5), membrane fraction (lane 6), soluble fraction (lane 7), and rHmuR purified using Ni-chelate chromatography (lane 8). The positions of molecular size markers (in kilodaltons) are on the left. (B) Identification of rHmuR. Whole-cell lysates and purified rHmuR were electrophoresed using SDS-PAGE and transferred onto a nitrocellulose membrane. *E. coli* harboring the vector alone (lane 1, uninduced; lane 2, induced), cells expressing rHmuR (lane 3, uninduced; lane 4, induced), inclusion bodies fraction (lane 5), membrane fraction (lane 6), soluble fraction (lane 7), rHmuR purified using Ni-chelate chromatography (lane 8). The positions of molecular size markers (in kilodaltons) are on the left. The immunoblot was probed with anti-fusion protein antibody and detected using chemiluminescence staining. (C) Hemoglobin binding by rHmuR. rHmuR purified by Ni-chelate chromatography was electrophoresed using SDS-PAGE and transferred onto a nitrocellulose membrane. The blot was probed with biotinylated human hemoglobin.

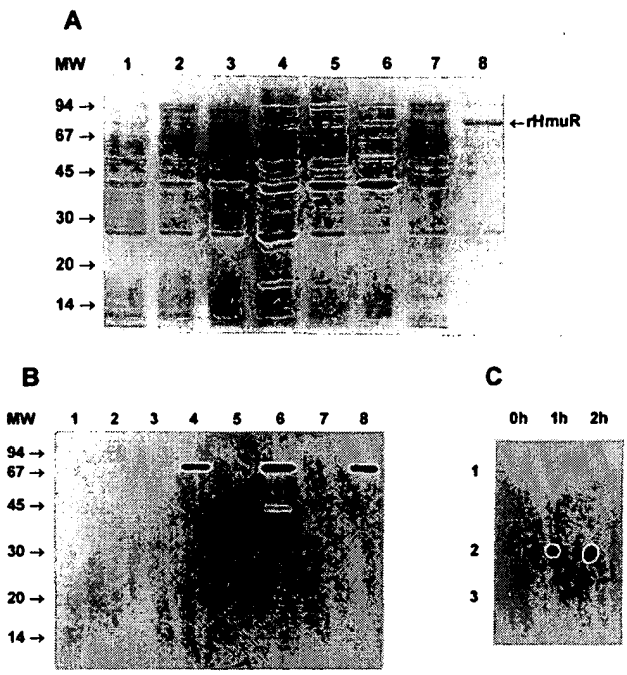

FIG. 7. Expression, purification, and surface exposure of membrane-bound rHmuR. (A) Expression of rHmuR. The gene encoding the protein with the signal peptide was cloned into pCRT7/CT-TOPO and expressed in *E. coli* BL21(DE3)pLysE. Lanes are designated in the same manner as in Fig. 6A. (B) Identification of rHmuR. Whole-cell lysates and purified rHmuR were electrophoresed using SDS-PAGE and transferred onto a nitrocellulose membrane. Lanes are designated in the same manner as in Fig. 6B. The immunoblot was probed with anti-fusion protein antibody and detected using chemiluminescence staining. (C) Identification of rHmuR on the surface of *E. coli* BL21(DE3)pLyE cells (panel 1, *E. coli* harboring vector alone; panel 2, *E. coli* expressing membrane-bound rHmuR; panel 3, *E. coli* expressing rHmuR deposited in inclusion bodies). The dot blot was probed with antibodies against the fusion protein using cells before and 1 and 2 h after IPTG induction.

amino acid sequence of membrane-bound rHmuR was determined by N-terminal sequencing of the protein by Edman degradation, after the transfer onto PVDF membranes of rHmuR purified by Ni-chelate chromatography. The resulting amino acid sequence was ANPPAQPT and matches 100% to the native HmuR following signal peptide cleavage (data not shown). Binding of hemoglobin and hemin by whole *E. coli* cells expressing rHmuR was examined using a spectrophotometric assay. As expected, only *E. coli* cells expressing membrane-bound rHmuR were found to bind hemoglobin and hemin (Fig. 8). We did not observe hemoglobin or hemin binding by *E. coli* cells in which rHmuR was deposited in inclusion bodies (Fig. 8) or by *E. coli* harboring the plasmid alone (data not shown). These results indicate that in *E. coli* BL21(DE3) pLysE, rHmuR is exported to the membrane, where it can bind both hemoglobin and hemin.

DISCUSSION

In this study we have determined that a newly identified TonB-dependent receptor, HmuR, is involved in the binding and utilization of hemoglobin and hemin in *P. gingivalis*. This is based on sequence analysis comparison, which reveals a high degree of homology of HmuR to TonB-dependent outer membrane receptors involved in the acquisition of iron from hemoglobin, characterization of the *P. gingivalis hmuR* mutant, and the ability of recombinant HmuR protein to bind hemoglobin and hemin. The *hmuR* gene containing its native signal peptide was used to express rHmuR, which was exported to the outer membrane in *E. coli* cells. We found that *E. coli* cells expressing rHmuR bound both hemoglobin and hemin. Using the *hmuR* gene without its native signal sequence allowed us to express and purify larger quantities of partially renatured rHmuR, and the purified protein was demonstrated to bind hemoglobin. Taken together, these results support the role of HmuR as a required *P. gingivalis* hemoglobin-hemin receptor.

In *H. influenzae*, the expression of the hemoglobin receptor HgpA is controlled by phase variation via strand slippage across "CCAA" repeats (33). Analysis of the *P. gingivalis hmuR* gene revealed the presence of 12 CCAA repeats at intervals of various lengths, suggesting that hemin-hemoglobin utilization via HmuR could be regulated by a similar mechanism. However, variability in the ability of *P. gingivalis* to utilize hemoglobin has not been examined, and it remains to be determined if hemin-hemoglobin utilization via HmuR in *P. gingivalis* is under phase variation.

The observation that the *hmuR* mutant did not exhibit a total lack in hemoglobin binding appears to be due to the

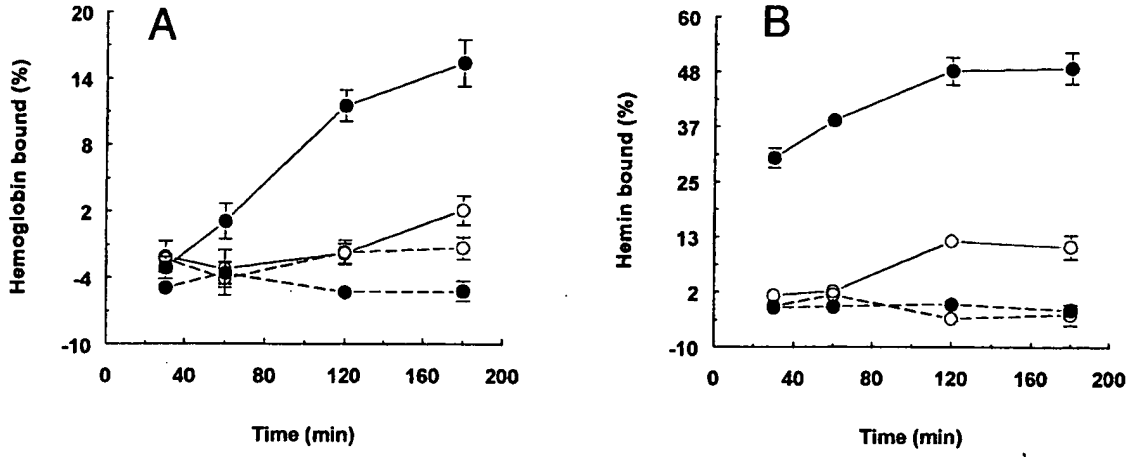

FIG. 8. Hemoglobin and hemin binding by *E. coli* expressing rHmuR. *E. coli* BL221(DE3)pLysE cells expressing rHmuR membrane bound (solid line) and rHmuR deposited in inclusion bodies (dotted line) grown in M9 media were harvested before (○) and after IPTG induction (●) and suspended in PBS. Human hemoglobin (A) and hemin (B) were added to final concentrations of 5 and 10 μM, respectively.

presence of intact *kgp* and *rgpA* genes in this strain. We have previously demonstrated that a *P. gingivalis kgp* mutant grows poorly with hemin or hemoglobin as sole iron sources (14). Studies in our laboratory have also demonstrated that soluble Kgp and HRgpA bind hemoglobin and that binding is mediated through the 40- and 44-kDa polypeptides of the Kgp and HRgpA complexes (Sroka et al., submitted), respectively. Likewise, hemoglobin binding to Kgp and HRgpA has also been reported by other investigators, although conflicting studies defining the region of the protein involved in hemoglobin binding have been reported (11, 21, 28; Sroka et al., submitted). Although Kgp can be found associated with the *P. gingivalis* outer membrane, at this point it appears premature to classify Kgp as a receptor. The amino acid sequence of Kgp has no similarity to TonB dependent outer membrane proteins. Rather Kgp may function as a soluble hemoglobin binding protein which, similar to hemophores, captures hemoglobin and delivers it to a second outer-membrane-associated receptor, possibly the hemoglobin receptor HmuR. The best characterized of the hemophore systems is that of the *S. marcescens* secreted protein, HasA, which extracts heme from hemoglobin and hemopexin-heme and delivers it to the outer membrane receptor HasR (17). Unlike siderophores, HasA is not internalized with its ligand during uptake. HasA has a very high affinity for heme; however, it is unclear how the heme is released from HasA onto HasR. Both apo HasA and holo HasA interact with HasR, indicating that HasA does not interact with HasR solely via the heme molecule. A similar extracellular hemin-binding protein (HasAp) has recently been described in *P. aeruginosa* (23).

We found that the *hmuR* mutant exhibited a decreased ability to grow with hemin and that *E. coli* cells expressing HmuR could bind hemin. We also demonstrated that hemoglobin can compete for the binding and accumulation of hemin in *P. gingivalis* (data not shown), further suggesting that hemin and hemoglobin transport can occur via a common pathway. Thus, in addition to its role in hemoglobin utilization, HmuR appears to function in hemin transport in *P. gingivalis*. Hemin binding in *P. gingivalis* has been observed to occur through both high- and low-affinity binding sites, and it has been proposed that this is mediated by separate outer membrane receptors (14). In addition to the TonB-dependent hemoglobin receptor, HmuR, *P. gingivalis* also appears to possess two additional putative TonB-dependent hemin receptors (HemR and Tla). It is possible that HemR and Tla could function to bind hemin directly; however, conclusive evidence for the roles of HemR and TlaA in hemin binding has not been reported. A *P. gingivalis tla* mutant was demonstrated to grow with high levels of hemin, but growth was decreased with low levels of this iron source. These results indicate that Tla is involved in hemin transport; however, it is not known if Tla functions in heme capture or in heme binding via a receptor-like mechanism. A definitive role for the *P. gingivalis* HemR protein in hemin transport has not been delineated since Karunakaran et al. (19) were unable to construct a *P. gingivalis hemR* mutant. Despite the fact that previous studies have determined that *hemR* is present in strains 53977, 381, and W50, we were unable to PCR amplify the *hemR* gene from *P. gingivalis* A7436 (data not shown), suggesting that in this strain hemin transport can occur independently of HemR. The *hmuR* gene was also found in *P. gingivalis* strains 381, 53977, and W50, with variability observed in the carboxy terminus of *hmuR* in these strains. This variability observed within the gene encoding the carboxy terminus of HmuR may be due to genomic rearrangements facilitated by *P. gingivalis* insertion sequence elements. Such rearrangements have recently been proposed to result in the variability in the *P. gingivalis* gingipain gene family (2, 29).

Our results also indicate that *hmuY* and *hmuR* are cotranscribed and that transcription is increased following growth of *P. gingivalis* in iron-limiting conditions. In a number of diverse microorganisms, genes involved in iron acquisition and virulence are transcriptionally regulated by the availability of iron through the Fur protein (3). Fur forms a dimer with ferrous iron and binds to a 19-bp DNA sequence (Fur box), which overlaps the promoters of iron-regulated genes, resulting in the inhibition of transcription. Upstream of the *P. gingivalis hmuY* start site we identified a region with homology to the Fur consensus binding sequence. The recent isolation of a *P. gingivalis fur* homolog (C. A. Genco and W. Simpson, unpublished data), together with the identification of a Fur box upstream of the *hmuY-hmuR* operon supports the role of Fur-mediated transcriptional control of the *P. gingivalis hmuR* gene. Interestingly, we found that the increased transcription of *hmuR* under iron-limiting conditions also correlated with an increase in hemoglobin binding of *P. gingivalis* whole cells. We found that hemoglobin binding increased fourfold when *P. gingivalis*

was grown in the presence of the iron chelator, dipyridyl (data not shown). Amano et al. (2) previously reported that hemoglobin binding to *P. gingivalis* whole cells is directly correlated with the successive passage of bacteria in media devoid of added heme. Thus, the increased hemoglobin binding of *P. gingivalis* whole cells obtained from cultures grown under iron limitation appears to result from the derepression of the *hmuR* gene as a result of Fur-mediated regulation. In contrast to our results, Karunakaran et al. (19) demonstrated that in *P. gingivalis* 53977, ORF1 (*hmuY*) was upregulated in the presence of hemin, while *hemR* was negatively regulated by hemin. In addition, these investigators demonstrated that ORF1 was part of a 1-kb transcript, while *hemR* was part of a 3-kb transcript. The differences in these findings may be due to the fact that *hmuR* and *hemR* are different genes and are regulated by different mechanisms or to strain-related differences in transcriptional regulation.

While our results indicate that HmuR is required for the binding and utilization of hemin and hemoglobin by *P. gingivalis*, little is known concerning the role(s) of other proteins in the transport of iron from these compounds. A search of the *P. gingivalis* W83 TIGR database allowed us to identify a putative hemin transport operon in *P. gingivalis* which exhibits a high degree of homology to the *Y. enterocolitica* hemin transport system. The *Y. enterocolitica* hemin-degrading protein HemS, hemin-binding protein HemT, hemin permease HemU, and ATP-binding hydrophilic protein HemV demonstrated homologies of 43, 48, 44, and 53%, respectively, with specific contigs in the *P. gingivalis* W83 database of the TIGR (41). While we recognize that the functions of these genes in *P. gingivalis* have not been defined, we postulate that the proteins they encode may function together with HmuR for the transport of hemin and heme from hemoglobin.

In summary, we have characterized the structural gene for a novel *P. gingivalis* TonB-dependent outer membrane receptor (HmuR) which functions both in hemoglobin and hemin binding and utilization in *P. gingivalis*. We demonstrated that the *hmuY* gene is found directly upstream of *hmuR*, that *hmuY* and *hmuR* are cotranscribed, and that transcription was negatively regulated by iron. Furthermore, recombinant HmuR was shown to bind hemoglobin, and *E. coli* cells expressing rHmuR were able to bind hemoglobin and hemin. We propose, based on these results, that HmuR serves as the major TonB-dependent outer membrane hemoglobin-hemin receptor in *P. gingivalis*. Future studies are aimed at defining the interaction between the HmuR and hemoglobin, hemin, and other substrates.

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Binding Specificity of the *Porphyromonas gingivalis* Heme and Hemoglobin Receptor HmuR, Gingipain K, and Gingipain R1 for Heme, Porphyrins, and Metalloporphyrins

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Previous genetic and biochemical studies have confirmed that hemoglobin and hemin utilization in *Porphyromonas gingivalis* is mediated by the outer membrane hemoglobin and heme receptor HmuR, as well as gingipain K (Kgp), a lysine-specific cysteine protease, and gingipain R1 (HRgpA), one of two arginine-specific cysteine proteases. In this study we report on the binding specificity of the recombinant *P. gingivalis* HmuR protein and native gingipains for hemoglobin, hemin, various porphyrins, and metalloporphyrins as assessed by spectrophotometric assays, by affinity chromatography, and by enzyme-linked immunosorbent assay. Protoporphyrin, mesoporphyrin, deuteroporphyrin, hematoporphyrin, and some of their iron, copper, and zinc derivatives were examined to evaluate the role of both the central metal ion and the peripheral substituents on binding to recombinant HmuR and soluble gingipains. Scatchard analysis of hemin binding to *Escherichia coli* cells expressing recombinant membrane-associated six-His-tagged HmuR yielded a linear plot with a binding affinity of 2.4×10^{-5} M. Recombinant *E. coli* cells bound the iron, copper, and zinc derivatives of protoporphyrin IX (PPIX) with similar affinities, and approximately four times more tightly than PPIX itself, which suggests that the active site of HmuR contains a histidine that binds the metal ion in the porphyrin ring. Furthermore, we found that recombinant HmuR prefers the ethyl and vinyl side chains of the PPIX molecule to either the larger hydroxyethyl or smaller hydrogen side chains. Kgp and HRgpA were demonstrated to bind various porphyrins and metalloporphyrins with affinities similar to those for hemin, indicating that the binding of Kgp and HRgpA to these porphyrins does not require a metal within the porphyrin ring. We did not detect the binding of RgpB, the arginine-specific cysteine protease that lacks a C-terminal hemagglutinin domain, to hemoglobin, porphyrins, or metalloporphyrins. Kgp and HRgpA, but not RgpB, were demonstrated to bind directly to soluble recombinant six-His-tagged HmuR. Several possible mechanisms for the cooperation between outer membrane receptor HmuR and proteases Kgp and HRgpA in hemin and hemoglobin binding and utilization are discussed.

Passive heme uptake through the outer membranes of gram-negative bacteria is not a significant route of heme entry (24, 33), and most bacteria possess specific heme uptake systems to use this compound as either an iron or iron-porphyrin source (reviewed in reference 20). In most gram-negative bacteria heme utilization is mediated by specific outer membrane receptors that bind directly to host heme-sequestering proteins. Several gram-negative bacteria also produce extracellular heme-binding proteins (hemophores). These secreted proteins extract heme from hemoglobin and deliver it to an outer membrane-associated protein, which transports heme into the cell. The best-characterized system is that of *Serratia marcescens* secreted protein HasA, which captures heme and hemoglobin and delivers it to outer membrane receptor HasR (21, 28).

Porphyromonas gingivalis, the etiological agent of adult periodontal disease, requires hemin for growth (17, 46). The binding and utilization of hemoglobin (2, 16, 25, 52) and hemin (5, 17, 54) have been demonstrated in *P. gingivalis* and related species. *P. gingivalis* expresses several outer membrane proteins in response to iron and heme limitation (5, 51); however, the role of these proteins in heme transport is not well defined. Several reports have also described *P. gingivalis* genes *hemR*, *ihtA*, and *tlr*, which exhibit homology to genes encoding TonB-dependent receptors (13, 23, 49). A role for the protein products of the *P. gingivalis hemR*, *ihtA*, and *tlr* genes has not been delineated because the respective *P. gingivalis* mutants have not been isolated.

We have described a *P. gingivalis* heme and hemoglobin receptor (heme/hemoglobin receptor; HmuR) which has homology with TonB-dependent outer membrane hemoglobin/heme receptors (50). *P. gingivalis hmuR* mutant cells bound less hemoglobin and hemin than did the parental strain and exhibited diminished growth with hemoglobin or hemin (50). Furthermore, we demonstrated that recombinant HmuR expressed in *E. coli* bound hemin and hemoglobin (50). Amino acid comparisons of the conserved motifs of several different hemoglobin/heme receptors and the *P. gingivalis* HmuR protein revealed that HmuR contains highly conserved domains containing invariant histidine residues (His95 and His434), glutamic acid residues (Glu448 and Glu458), and the FRAP (in HmuR YRAP) and NPNL (in HmuR NPDL) amino acid boxes, which may be involved in hemoglobin and heme binding (4, 50). It was previously shown that the *hemR* gene is identical with the *hmuR* gene in the N-terminal portion but that these two genes differ in their C termini (23, 50). Despite the fact

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TABLE 1. Primers used in this study

Primer[a] (sequence)	Description
F1 (ATGGCCAACCCTCCGGCCCAACCTA) R1 (GAAAGTGATCCGAACCAACCCGTAT)	Amplifies the *hmuR* gene without the signal peptide sequence and without the stop codon
F2 (ATGAAAAGTCTAGTAACAAAGCAGG) R1 (GAAAGTGATCCGAACCAACCCGTAT)	Amplifies the *hmuR* gene with the signal peptide sequence and without the stop codon
F1 (ATGGCCAACCCTCCGGCCCAACCTA) R2 (TTAGAAAGTGATCCGAACCAACCCGTAT)	Amplifies the *hmuR* gene without the signal peptide sequence and with the stop codon
F2 (ATGAAAAGTCTAGTAACAAAGCAGG) R2 (TTAGAAAGTGATCCGAACCAACCCGTAT)	Amplifies the *hmuR* gene with the signal peptide sequence and with the stop codon

[a] F, forward; R, reverse.

that previous studies have determined that *hemR* is present in strains 53977, 381, and W50, we were unable to amplify the *hemR* gene from *P. gingivalis* A7436, suggesting that in this strain hemin transport can occur independently of HemR.

In addition to conventional outer membrane receptors, heme and hemoglobin utilization in *P. gingivalis* also requires participation of the cysteine proteases referred to as gingipains (12, 19, 26). The gingipains exhibit proteolytic enzymatic activity against a range of host proteins including host proteinase inhibitors, immunoglobulins, iron-sequestering proteins, extracellular matrix proteins, bactericidal proteins and peptides, and proteins involved in the coagulation, complement, and kallikrein/kinin cascades (15, 22, 31, 45). *P. gingivalis* lysine-specific gingipain K (Kgp) and arginine-specific gingipain R1 (HRgpA) are purified as noncovalent complexes of the catalytic domain associated with four polypeptide chains derived from the hemagglutinin domain (3, 11, 36, 37, 40, 41, 42). These gingipains occur either in extracellular soluble or in membrane-associated forms (40, 41). In contrast to Kgp and HRgpA, a second arginine-specific gingipain, R2 (RgpB), contains only a catalytic domain (44) and is not required for hemoglobin and heme utilization in *P. gingivalis*. Previous studies have reported that different portions of Kgp and HRgpA can bind hemoglobin, hemin, and protoporphyrin IX (PPIX) (14, 25, 35, 38, 48). Kgp has also recently been demonstrated to degrade hemoglobin (29, 53), hemopexin, haptoglobin (53), and transferrin (6). Genetic analysis has confirmed a role for Kgp in hemoglobin and hemin utilization in *P. gingivalis* (18, 51). It has recently been proposed that soluble Kgp and outer membrane receptor HmuR function together for the transport of hemin from hemoglobin in *P. gingivalis* (20).

In this study we report on the binding specificity of recombinant *P. gingivalis* HmuR and native soluble gingipains for hemin, hemoglobin, porphyrins, and metalloporphyrins. A series of porphyrins and metalloporphyrins were chosen to evaluate the role of both the central metal ion and the peripheral substituents in porphyrin binding to recombinant HmuR and native gingipains. Our results demonstrate that outer membrane receptor HmuR binds heme and related metalloporphyrins more tightly than hemoglobin. The iron, copper, and zinc derivatives of PPIX bound to recombinant HmuR with similar affinities, and more tightly than PPIX itself, suggesting that the active site of HmuR has a histidine that binds to the metal ion present in the porphyrin ring. Native Kgp and HRgpA bound selected porphyrins approximately as well as the corresponding metalloporphyrins, indicating that the binding of Kgp and HRgpA to these compounds does not require a metal present in the porphyrin ring. RgpB, which is missing the C-terminal hemagglutinin domain present in Kgp and HRgpA, did not bind to these compounds. Finally, we demonstrate that soluble Kgp and HRgpA, but not RgpB, bind directly to recombinant HmuR.

MATERIALS AND METHODS

Construction of HmuR expression plasmids. The *hmuR* gene was amplified from total genomic DNA of *P. gingivalis* strain A7436 as previously reported (50). Briefly, the forward primer (Table 1) was designed to produce *hmuR* either without (F1) or with (F2) its native signal peptide sequence. The reverse primer was designed to remove the native stop codon (R1) to preserve the reading frame through the C-terminal tag. To obtain recombinant HmuR without the tag consisting of the V5 epitope and six histidines (V5–six-His tag), the stop codon was included (R2). The amplified products were purified and cloned into vector pCRT7/CT-TOPO (Invitrogen, Carlsbad, Calif.), coding for the V5 epitope and polyhistidine (six-His) regions. The resulting plasmids (pTO1, pTO2, pTO3, and pTO4; Table 2) were then transformed into *Escherichia coli* strain TOP10F′ (Invitrogen). Transformants were selected on Luria-Bertani plates containing 100 μg of ampicillin/ml, and the insertion was confirmed by restriction enzyme analysis, PCR, and sequence analysis.

Expression and purification of recombinant HmuR. *E. coli* strains BL21(DE3) pLysS and BL21(DE3)pLysE (Invitrogen) were transformed with either pTO1, pTO2, pTO3, pTO4, or pCRT7/CT-TOPO and grown overnight at 37°C in minimal medium (M9) supplemented with 100 μg of ampicillin and 34 μg of chloramphenicol/ml. Overnight bacterial cultures were inoculated into fresh M9 medium and grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.5 to 0.6. Expression of the *P. gingivalis hmuR* gene was induced by the addition of 0.5 to 1 mM isopropyl β-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, Mo.), followed by a 3- to 5-h growth period. After IPTG induction, *E. coli* BL21(DE3) pLysS and BL21(DE3)pLysE cells expressing TO1 were harvested by centrifugation for 20 min at 8,000 × *g*. Recombinant HmuR containing the V5–six-His tag (rHmuR-6His) and lacking the signal sequence was purified from inclusion bodies using Ni-chelate chromatography under denaturing conditions as described previously (50). The purified protein was dialyzed to decreasing concentrations of urea and finally to 0.5 M urea made in 20 mM phosphate buffer, pH 7.4, containing 0.14 M NaCl (phosphate-buffered saline [PBS]) and 0.1% *n*-octyl-β-D-glucopyranoside (OG; Sigma). After dialysis less than 10% of the rHmuR-6His was susceptible to renaturation and present in the soluble fraction, as determined by protein concentration. The remainder of the rHmuR-6His protein obtained after the final dialysis was present in the denatured nonsoluble fraction.

To localize recombinant HmuR, total membrane fractions were isolated from *E. coli* cells (adjusted to an OD_{600} of 1.0) harboring pTO2, pTO3, or the vector alone after centrifugation (70,000 × *g*, 1 h) of the supernatant remaining after the first centrifugation at 8,000 × *g* for 20 min. Total-membrane fractions were solubilized in 0.5% sarcosyl (Sigma) in PBS containing protease inhibitors, and after centrifugation (100,000 × *g*, 1 h) outer membrane fractions were collected. Detection of the recombinant HmuR was performed after polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) using Coomassie brilliant blue G-250 (CBB; Invitrogen) staining or after transfer onto

TABLE 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
P. gingivalis A7436	Wild type	Laboratory collection
E. coli TOP10F′	F′ {*lacI*^{q}/Tn*10* (Tet^r)} *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
E. coli BL21 (DE3)pLysE	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)pLysE(Cam^r)	Invitrogen
E. coli BL21 (DE3)pLysS	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)pLysS(Cam^r)	Invitrogen
pCRT7/CT-TOPO	Amp^r	Invitrogen
pTO1	pCRT7/CT-TOPO containing the *hmuR* gene without the signal peptide sequence and with the fusion tag	50
pTO2	pCRT7/CT-TOPO containing the *hmuR* gene with the signal peptide sequence and with the fusion tag	50
pTO3	pCRT7/CT-TOPO containing the *hmuR* gene with the signal peptide sequence and without the fusion tag	This study
pTO4	pCRT7/CT-TOPO containing the *hmuR* gene without the signal peptide sequence and without the fusion tag	This study

nitrocellulose membranes by probing with monoclonal anti-V5 antibodies (Invitrogen) or polyclonal anti-HmuR antibodies (Lampire Biological Laboratories, Pipersville, Pa.). The latter were raised to the rHmuR-6His purified from inclusion bodies. The immunoglobulin G (IgG) fraction was isolated from the resulting antiserum using a HiTrap protein A affinity column (Pharmacia Biotech, Piscataway, N.J.). After incubation with a secondary antibody conjugated with horseradish peroxidase (HRP; Sigma) chemiluminescence staining was used to detect the complexes formed (50).

UV-Vis spectrophotometric analysis. Heme binding to rHmuR-6His purified from inclusion bodies was monitored by UV-visual (UV-Vis) absorption analysis on a Beckman DU 7500 spectrophotometer scanning from 200 to 800 nm. The final mixture of 5 μM rHmuR-6His (renatured by dialysis against PBS, pH 7.4, containing 0.1% OG and 0.5 M urea) with 20 μM hemin (dissolved in dimethyl sulfoxide [DMSO]; final DMSO concentration in the mixture was 1 to 10%) was placed in a cuvette (0.5-cm cell length), and spectra were recorded immediately after mixing (time zero) and after 5, 15, 30, and 60 min of incubation at room temperature (RT). Nonrenatured rHmuR-6His, present after dialysis in the denatured nonsoluble fraction, was dissolved in PBS containing 0.1% OG and 8 M urea and was also examined by UV-Vis spectrophotometric analysis for hemin binding. The UV-Vis analyses of serum protein complexes with hemoglobin or hemin were performed under similar conditions as described above, with the exception that PBS was used to dissolve and dilute all proteins.

Binding of *E. coli* cells expressing recombinant HmuR to hemoglobin, porphyrins, and metalloporphyrins. *E. coli* BL21(DE3)pLysS and BL21(DE3) pLysE cells expressing rHmuR-6His (containing the V5-six-His tag) or rHmuR (V5-six-His tag not produced) deposited in inclusion bodies (lacking the signal sequence) or membrane associated (containing the signal sequence) and cells containing the vector alone were grown in M9 medium and harvested before and after IPTG induction. The cells were washed with PBS and adjusted to an OD_{600} of 1.0, and 0.8-ml aliquots of the cell suspension in PBS were mixed with 0.2 ml of hemoglobin (5 μM) or hemin (10 μM) or other porphyrins or metalloporphyrins (10 μM). To reduce self-aggregation of hemin and other metalloporphyrins or porphyrins, DMSO (1 to 10%) was included in all assays (10, 20). PPIX and mesoporphyrin (MPIX) were obtained from Aldrich Biochemicals (Milwaukee, Wis.); iron(III) α,β,γ,δ-tetraphenylporphine tetrasulfonic acid ($FeTPPS_4$), hematoporphyrin (HPIX), deuteroporphyrin (DPIX), the copper derivative of MPIX (CuMPIX), and the zinc and copper derivatives of PPIX (ZnPPIX, and CuPPIX, respectively) were obtained from Porphyrin Products (Logan, Utah); the iron and zinc derivatives of MPIX (FeMPIX and ZnMPIX, respectively) were obtained from Midcentury (Posen, Ill.); hemoglobin and hemin were obtained from Sigma. Samples were incubated at RT for 1 h and centrifuged, and the OD_{400} of the supernatant was measured. Compounds diluted in PBS were incubated under the same conditions and served as appropriate controls. The binding of all compounds was determined by the decrease of absorbance of the supernatant compared to those for control samples, which were set as 100%. For the saturation of hemin binding to rHmuR-6His, nonspecific hemin binding to *E. coli* cells harboring the vector alone was subtracted from hemin binding to *E. coli* cells expressing rHmuR-6His.

Purification of gingipains. Kgp, HRgpA, and RgpB were purified from *P. gingivalis* strain HG66 cultures as previously described (42, 44) and were kindly provided by Jan Potempa (Jagiellonian University, Cracow, Poland). Approximately 5 mg of each gingipain from 1 liter of bacterial culture was purified to homogeneity as determined by SDS-PAGE and CBB staining and, after transfer onto a nitrocellulose membrane, by reactivity with the appropriate antibodies. In addition, the concentration of active gingipain in each batch was determined by active-site titration using specific inhibitors *Z*-Phe-Lys-2,4,6-trimethylbenzoyloxymethylketone (FKck) and H-D-Phe-Phe-Arg-chloromethylketone (FFRck) (Bachem Biosciences Inc., King of Prussia, Pa.) for gingipain K and gingipain R, respectively (43). Antibodies to each purified gingipain (Lampire Biological Laboratories) were produced as previously described (19). IgG fractions were purified from the antisera using a HiTrap protein A affinity column (Pharmacia Biotech), and their specificities and activities were confirmed.

Binding of recombinant HmuR to hemoglobin, gingipains, and serum proteins. Binding of rHmuR-6His to hemoglobin, gingipains, and serum proteins was examined by an enzyme-linked immunosorbent assay (ELISA). Six-His-tagged rHmuR purified from inclusion bodies was immobilized (0.05 nmol per well) onto the surface of Ni-nitrilotriacetic acid (NTA) HisSorb wells (Qiagen, Valencia, Calif.) and incubated overnight at 4°C. This was followed by the addition of hemoglobin, haptoglobin (Sigma), transferrin saturated with iron (Sigma), human serum albumin (HSA; Sigma), hemopexin (Sigma), Kgp, HRgpA, or RgpB (0.005 to 1.0 nmol per well). We also prepared complexes of hemoglobin with haptoglobin, hemopexin with hemin, and HSA with hemin by incubation of serum proteins with hemoglobin or hemin in a molar ratio of 1:1 for 1 h at 37°C. The formation of these complexes was confirmed by UV-Vis analysis at 200 to 800 nm. Free hemoglobin or hemin was not observed in these preparations. The complexes formed between rHmuR-6His and the proteins were detected by probing with specific antibodies to each serum protein (goat antihemoglobin, antihaptoglobin, antihemopexin, rabbit anti-HSA, and antitransferrin diluted 1:5,000, 1:10,000, 1:10,000, 1:5,000, and 1:5,000, respectively; Biomeda, Foster City, Calif., or Sigma) or gingipain (rabbit anti-Kgp, -HRgpA, and -RgpB diluted 1:30,000, 1:20,000, and 1:10,000, respectively), followed by appropriate secondary antibodies conjugated with HRP. Substrate *o*-phenylenediamine (Sigma) was added to wells and incubated at RT for 15 to 30 min. The reactions were stopped by addition of 50 μl of 12.5% sulfuric acid, and the absorbance was measured at 490 nm.

Binding of recombinant HmuR and gingipains to hemoglobin- or heme-agarose. Hemoglobin- and heme-agarose beads (Sigma) were washed with PBS or PBS containing 0.1% OG, resuspended in 20 μl of the buffer, and incubated with agitation overnight at 4°C with rHmuR-6His purified from inclusion bodies (0.01 nmol of rHmuR-6His per sample) and Kgp, HRgpA, or RgpB (0.01 nmol of Kgp and HRgpA and 0.02 nmol of RgpB; gingipains contained FKck and FFRck as inhibitors) in a total volume of 50 μl. Hemoglobin-agarose contained 6.1 to 12.3 nmol of hemoglobin per sample (0.02 to 0.05 μmol of heme in a hemoglobin sample), and heme-agarose contained 0.08 to 0.16 μmol of heme per sample. After centrifugation, the supernatant fluids were collected and the conjugated agarose beads were washed three times with PBS or PBS containing 0.1% OG. All samples were then boiled in Laemmli sample buffer and after centrifugation were subjected to SDS-PAGE. Gels were either stained with CBB or transferred onto nitrocellulose membranes. Recombinant HmuR was detected on blots with an anti-V5-HRP antibody (Invitrogen), and the other proteins were detected with the appropriate antibodies. Chemiluminescence detection was performed as previously described (50).

Binding of gingipains to hemoglobin and various porphyrins and metalloporphyrins. Binding of Kgp, HRgpA, and RgpB to hemoglobin, hemin, porphyrins, and metalloporphyrins was studied using an ELISA. Polystyrene plates (Dynex, Chantilly, Va.) were coated overnight at 4°C with 100 μl of 5 μM hemoglobin or

10 μM porphyrins or metalloporphyrins per well. Human hemoglobin was dissolved in PBS, whereas all other compounds were dissolved in DMSO and then diluted with PBS (final DMSO concentration of 1 to 10%). Nonspecific binding sites were blocked overnight at 4°C with 1% bovine serum albumin (BSA)-PBS, followed by addition of 0.01 to 2 nmol of Kgp, HRgpA, or RgpB (containing the appropriate inhibitors) in 100 μl of 1% BSA-PBS per well. Bound gingipains were detected by rabbit anti-Kgp, -HRgpA, or -RgpB antibodies diluted 1:30,000, 1:20,000, and 1:10,000, respectively, and by goat anti-rabbit IgG antibodies conjugated with HRP, diluted 1:10,000. All reactions were performed for 1 h at 37°C. Washing was performed with PBS or PBS containing 0.1% Tween 20. The color was developed as described above.

Statistical analysis. Data expressed as means ± standard deviations (SD) were analyzed using Student's *t* test; *P* values below 0.05 were considered significant.

RESULTS

Recombinant HmuR-hemin spectra and saturation of hemin binding to membrane-associated rHmuR-6His. In a previous study we demonstrated that recombinant HmuR containing its native signal peptide sequence and the V5–six-His tag (rHmuR-6His) was exported and associated with the outer membrane of *E. coli* cells (50). We also demonstrated that *E. coli* cells expressing membrane-bound rHmuR-6His bound hemoglobin and hemin (50). However, the binding specificity of rHmuR-6His for these ligands has not been examined in detail.

We examined the localization of the recombinant HmuR lacking the V5–six-His tag (rHmuR) and compared its expression and localization with those of rHmuR-6His. As shown in Fig. 1A and B the association of rHmuR and that of rHmuR-6His with the outer membranes of *E. coli* cells and expression levels of both proteins were comparable. We also determined that the addition of the His tag did not significantly affect the ability of *E. coli* cells expressing recombinant HmuR to bind hemin (Fig. 1C). As expected, only *E. coli* cells expressing membrane-associated rHmuR-6His (containing the His tag) or rHmuR (lacking the His tag) were found to bind hemin. In contrast, *E. coli* cells in which rHmuR-6His and rHmuR both lacked the signal sequences and after expression were deposited in inclusion bodies bound small amounts of hemin (Fig. 1C). These results indicate that *E. coli* cells expressing either rHmuR-6His or rHmuR can bind hemin and that the His tag did not significantly affect this binding.

The ability of rHmuR-6His purified from inclusion bodies to bind heme was examined by incubating the soluble protein with hemin and measuring the absorbance of hemin at 200 to 800 nm. Following the addition of rHmuR-6His (5 μM) to a freshly prepared hemin solution (20 μM) the absorption spectrum of heme in the Soret region changed from the spectrum of free heme (380 nm) to the typical spectrum of a heme-protein complex (408 nm) (Fig. 2). Spectral changes of rHmuR-6His with hemin were essentially complete after a 15-min incubation. This shift in the wavelength of the absorbance reflects a modification of the heme absorption spectrum caused by the binding of hemin to rHmuR-6His. We also found that rHmuR-6His purified from inclusion bodies but nonrenatured and dissolved in 8 M urea did not bind hemin (Fig. 2).

The saturation of hemin binding by membrane-associated rHmuR-6His expressed in *E. coli* cells was further examined by a spectrophotometric assay. Binding of hemin to whole *E. coli* cells was expressed as a decrease in the absorbance of the supernatant samples at 400 nm compared to that of samples

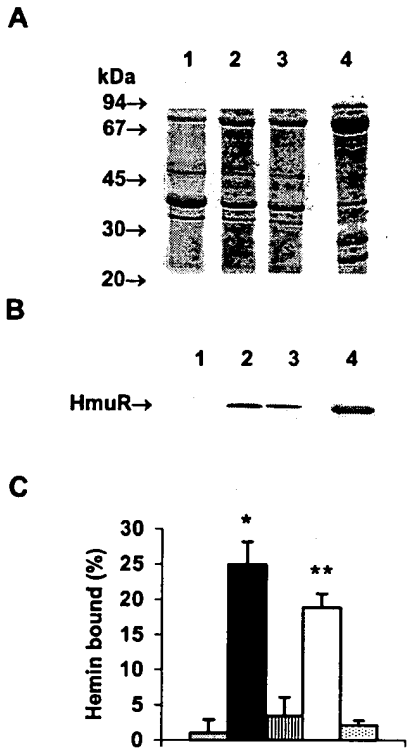

FIG. 1. *E. coli* cells expressing rHmuR-6His (containing the signal sequence and the V5–six-His tag) and rHmuR (containing the signal sequence but lacking the V5–six-His tag) bind hemin. (A and B) Localization of recombinant HmuR in *E. coli* cells. Membrane fractions of *E. coli* cells harboring the vector alone (lane 1), expressing rHmuR (lane 2), expressing rHmuR-6His (lane 3), and expressing rHmuR-6His lacking the signal sequence and deposited in inclusion bodies (lane 4) were adjusted to an OD_{600} of 1.0, and outer membranes (lanes 1 to 3) and inclusion bodies (lane 4) were isolated. (A) SDS–12.5% PAGE gel stained with CBB (10-μl portions of the samples were loaded onto the gel). (B) Western blot developed by probing with anti-HmuR antibodies raised to the recombinant six-His-tagged protein (5-μl portions of the samples were loaded onto the gel). (C) Hemin binding to *E. coli* cells expressing rHmuR. *E coli* cells were resuspended in PBS, adjusted to an OD_{600} of 1.0, and incubated for 1 h at RT with 10 μM hemin. Binding was determined by the decrease in the absorbance of the supernatant at 400 nm and was recorded as the percentage of the input hemin. Three independent experiments were performed in duplicate. Data are means ± SD. Asterisk, $P < 0.001$ for *E. coli* expressing membrane-associated rHmuR-6His (black bar) versus *E. coli* harboring the vector alone (grey bar); double asterisks, $P < 0.05$ for *E. coli* expressing membrane-associated rHmuR (open bar) versus *E. coli* harboring the vector alone. *E. coli* expressing rHmuR-6His (striped bar) and rHmuR (dotted bar) lacking the signal sequence, both of which were deposited in inclusion bodies, is also shown.

containing only hemin (Fig. 3A). Scatchard analysis of hemin binding to rHmuR-6His yielded a linear plot with a binding affinity (K_d) of 2.4×10^{-5} M (Fig. 3B). *E. coli* cells containing the vector alone bound hemin nonspecifically with very low efficiency. This binding did not show saturation, and the transformed data clustered around the origin (data not shown). These results demonstrate that *E. coli* cells with recombinantly expressed HmuR can bind hemin.

Binding of rHmuR-6His to hemin and hemoglobin immobilized on agarose. The binding of recombinant HmuR to hemoglobin and hemin was also assessed using hemoglobin and

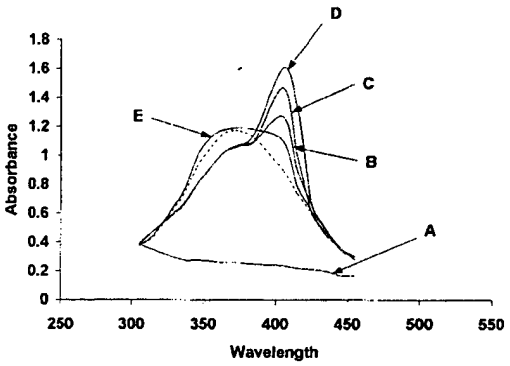

FIG. 2. Absorption spectra of hemin–rHmuR-6His complex. The absorption spectra in the Soret region of rHmuR-6His (5 μM) purified and renatured from inclusion bodies alone (A) or with added hemin (20 μM) were recorded immediately (B) and 5 (C) and 15 min (D) after mixing. (E) Absorption spectrum of hemin (20 μM) added to nonrenatured rHmuR-6His (5 μM) purified from inclusion bodies and dissolved in 8 M urea (recorded after 15 min of incubation with hemin). Dotted line, 20 μM hemin alone.

heme immobilized on agarose beads. The amounts of hemoglobin and heme bound to agarose exceeded the amounts of rHmuR-6His. We found that rHmuR-6His bound to heme-agarose (Fig. 4). When rHmuR-6His was incubated with hemoglobin-agarose, a larger amount of nonbound recombinant protein was present in the flowthrough fraction than in the heme-agarose flowthrough fraction (Fig. 4). These results confirmed that rHmuR-6His purified from inclusion bodies can bind to hemoglobin and hemin and suggested that binding to hemin can be more efficient than binding to hemoglobin. Similar results were obtained using membrane-associated rHmuR-6His. *E. coli* cells expressing rHmuR-6His bound lower levels of hemoglobin than of hemin (see Fig. 6).

***E. coli* cells expressing membrane-associated rHmuR-6His bind porphyrins and metalloporphyrins.** We used a spectrophotometric assay to assess the ability of whole *E. coli* cells expressing membrane-associated rHmuR-6His to bind hemoglobin, porphyrins, and metalloporphyrins. We compared the binding of hemin with the corresponding zinc and copper metalloporphyrins (ZnPPIX and CuPPIX), as well as PPIX itself

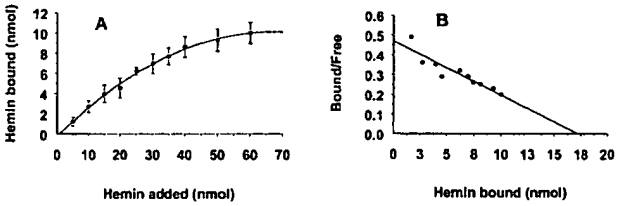

FIG. 3. Saturation of hemin binding to *E. coli* cells expressing membrane-associated rHmuR-6His. Saturability of hemin binding (A) and Scatchard plot analysis (B) of *E. coli* expressing recombinant HmuR are shown. *E coli* cells were resuspended in PBS and incubated for 1 h at RT with hemin. Binding was determined by the decrease of absorbance of the supernatant at 400 nm and recorded as the percentage of the input hemin. Nonspecific hemin binding to *E. coli* cells harboring the vector alone was subtracted from hemin binding to *E. coli* cells expressing rHmuR-6His. Two independent experiments were performed in duplicate. Data are means ± SD.

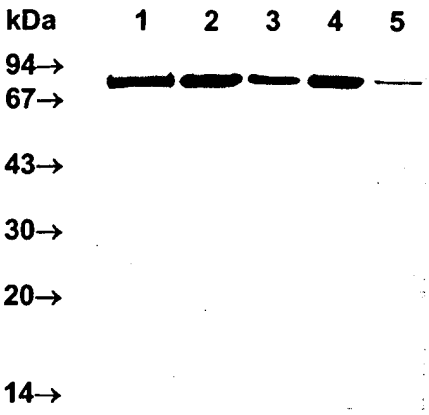

FIG. 4. Binding of rHmuR-6His to hemoglobin or heme immobilized on agarose. Recombinant HmuR-6His purified from inclusion bodies (0.01 nmol per sample) was incubated with 20 μl of hemoglobin-agarose (6.1 to 12.3 nmol of hemoglobin per sample; 0.02 to 0.05 μmol of heme in hemoglobin sample) or heme-agarose (0.08 to 0.16 μmol of heme per sample). Samples were then separated by SDS-PAGE and detected using CBB staining (lane 1) or with the anti-V5 antibody (lanes 2 to 5). Lane 1, soluble rHmuR-6His purified and renatured from inclusion bodies; lanes 2 and 4, rHmuR-6His bound to hemoglobin (lane 2) or heme (lane 4); lanes 3 and 5, flowthrough fractions from hemoglobin-agarose (lane 3) and heme-agarose (lane 5).

(Fig. 5). The same series of metals were evaluated for MPIX, in which the vinyl groups on the heme periphery at the 2 and 4 positions are replaced by ethyl groups (Fig. 5). To determine if alterations at the 2 and 4 positions of the heme substantially affect binding, we utilized DPIX and HPIX, which have H- and $CHOHCH_3$, respectively, at the 2 and 4 positions (Fig. 5). *E. coli* cells expressing recombinant HmuR containing the His tag were used for these studies; in initial studies we found that the addition of the His tag did not significantly affect the ability of recombinant HmuR to bind hemin (Fig. 1C) and other

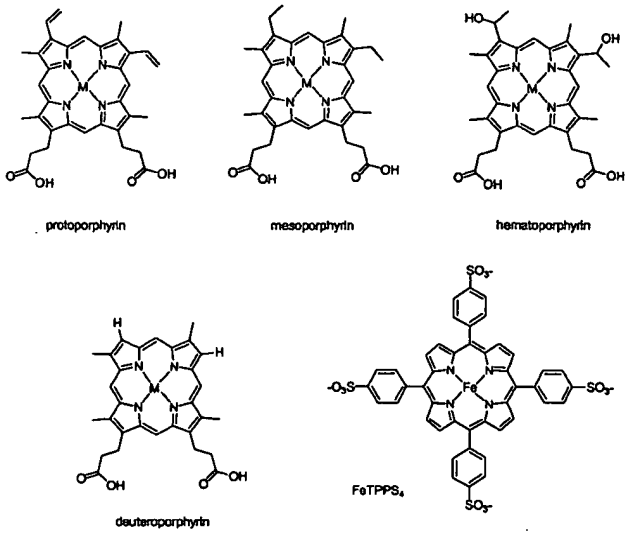

FIG. 5. Structures of the porphyrins and metalloporphyrins used. The compounds utilized to examine the binding specificity of HmuR and gingipains for heme are depicted. M, metal (Fe, Zn, or Cu) within the PPIX ring.

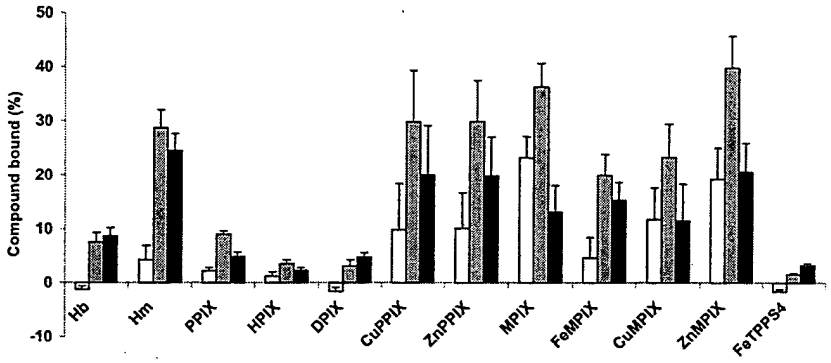

FIG. 6. *E. coli* cells expressing membrane-associated rHmuR-6His bind porphyrins and metalloporphyrins. Binding to *E. coli* expressing 6His-rHmuR (grey bars), and to control *E. coli* harboring the vector alone (open bars) is shown. Black bars, differences between the two data sets. *E. coli* cells were resuspended in PBS and incubated for 1 h at RT with hemoglobin (5 μM) or porphyrins or metalloporphyrins (10 μM). Binding was determined by the decrease of absorbance at 400 nm of the supernatant and recorded as the percentage of the input porphyrins. Data are means ± SD from three independent experiments performed in duplicate. Hb, hemoglobin; Hm, hemin.

metalloporphyrins (data not shown). The binding of all compounds was determined by the decrease in the absorbance of the supernatant compared to those for control samples, which were set as 100% (Fig. 6). Some of the porphyrins and metalloporphyrins showed substantial nonspecific binding to the control *E. coli* cells harboring the vector alone (Fig. 6). To account for this, the data were replotted for each compound in Fig. 6 as the difference between the two data sets. The discussion below refers to this difference for each compound.

The porphyrins themselves bound to *E. coli* expressing membrane-associated rHmuR-6His in the following order of affinity: MPIX > PPIX > DPIX ≈ HPIX. In the metalloprotoporphyrin series, *E. coli* expressing rHmuR-6His bound hemin, CuPPIX, and ZnPPIX with almost equal affinities, which were approximately four times higher than that for PPIX itself. The same series of metalloporphyrins with the MPIX skeleton was studied. In the MPIX series, there was substantial nonspecific binding; when this background was subtracted, all of the derivatives bound with the same affinity within experimental error.

We also examined $FeTPPS_4$, as it was previously reported to support the growth of *Vibrio vulnificus* as a single iron source (34). *E. coli* cells expressing rHmuR-6His bound $FeTPPS_4$ with very low efficiency; this binding was comparable to the nonspecific binding observed in control cells containing the vector alone (Fig. 6). Thus, the tetraphenylporphyrin structure makes this compound less accessible for HmuR than the other metalloporphyrins based on the natural porphyrin skeleton (Fig. 5).

These results demonstrate that *E. coli* cells expressing membrane-associated rHmuR-6His bind iron, copper, and zinc derivatives of PPIX more tightly than PPIX. This presumably suggests that the active site of HmuR has a histidine, which binds to the metal present in the porphyrin ring.

Binding of rHmuR-6His to serum proteins. Serum hemoglobin released from erythrocytes is tightly bound to haptoglobin, and heme is bound to hemopexin or albumin (20). Hemoglobin bound to haptoglobin and hemin complexed to hemopexin can be used as iron sources by *P. gingivalis*, indicating that this microorganism has a mechanism for removing the hemin from these host iron-binding proteins (29, 53). To determine if recombinant HmuR could bind haptoglobin, hemopexin, or albumin, we examined the ability of rHmuR-6His purified from inclusion bodies to bind these proteins by ELISA. As a negative control, we utilized iron-saturated transferrin. We did not detect binding of rHmuR-6His to serum apo-haptoglobin, albumin, apo-hemopexin, or transferrin saturated with iron (Fig. 7). As expected, binding of rHmuR-6His to hemoglobin was observed in this assay. Recombinant HmuR was also demonstrated to bind complexes of haptoglobin with hemoglobin, hemopexin with hemin, and albumin with hemin; however, the affinities were lower than that of hemoglobin.

Binding of gingipains to hemoglobin, porphyrins, and metalloporphyrins. We next examined the ability of gingipains to bind hemoglobin, porphyrins, and metalloporphyrins using ELISA, as described above for rHmuR-6His. We found that both Kgp and HRgpA bound to the porphyrins and metalloporphyrins tested with affinities similar to those for hemin (Fig. 8). The ability of these gingipains to bind metalloporphyrins and porphyrins similarly confirms that the binding of Kgp and HRgpA to these porphyrins does not require a metal present within the porphyrin ring. Interestingly, we observed greater binding of Kgp and HRgpA to hemoglobin than to the tested porphyrins and metalloporphyrins (Fig. 8). These observations

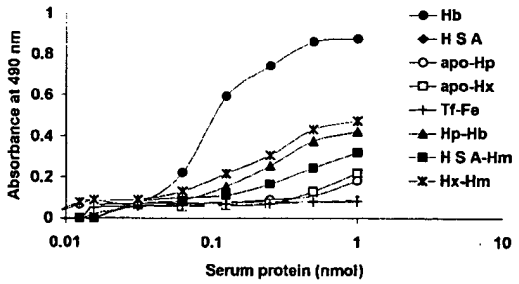

FIG. 7. Binding of rHmuR-6His to serum proteins. Ni-NTA plates were coated with rHmuR-6His purified and renatured from inclusion bodies (0.05 nmol per well) and incubated with human hemoglobin (Hb), haptoglobin (Hp), human serum albumin (HSA), hemopexin (Hx), transferrin saturated with iron (Tf-Fe), and complexes of Hp-Hb, Hx-Hm, and HSA-Hm (0.005 to 1.0 nmol of serum protein per well). The binding was detected using antibodies to the appropriate protein. Values are representative of two separate experiments performed in triplicate.

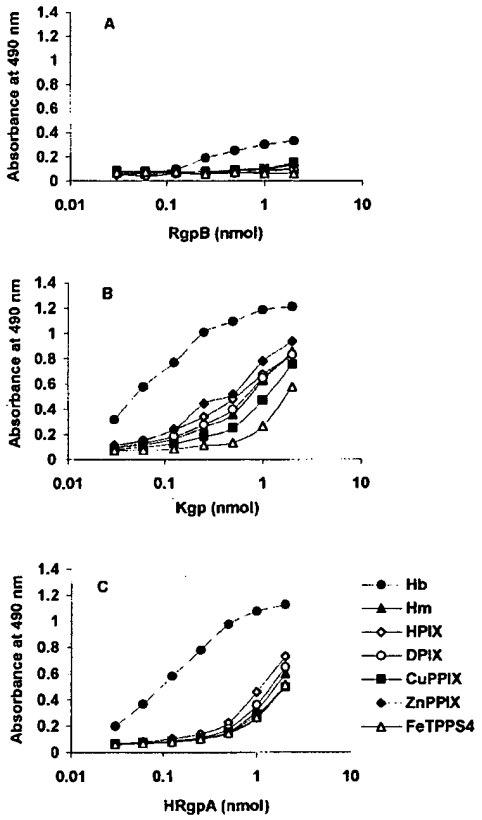

FIG. 8. Binding of gingipains to porphyrins and metalloporphyrins. Plates were coated with hemoglobin (Hb; 5 μM) or porphyrins or metalloporphyrins (10 μM). After incubation with RgpB (A), Kgp (B), or HRgpA (C) (0.01 to 1 nmol per well), binding was detected using antibodies to the appropriate protein. Values are representative of experiments performed in triplicate on three separate occasions. Hm, hemin.

suggest that substantial portions of the recognition sites of Kgp and HRgpA for hemoglobin are due to protein-protein interactions. In general, Kgp bound hemoglobin and the porphyrins and metalloporphyrins more efficiently than HRgpA. RgpB, which lacks the hemagglutinin domain, showed little or no binding to hemoglobin, hemin, and the porphyrins and metalloporphyrins (Fig. 8).

Gingipain binding to heme- and hemoglobin-agarose. To further examine the binding efficiency of the gingipains to hemoglobin and hemin, we utilized heme- and hemoglobin-agarose as described above for rHmuR-6His. We observed that Kgp and HRgpA bound to hemoglobin and heme immobilized on agarose (data not shown), confirming the results obtained by ELISA (Fig. 8). RgpB demonstrated little ability to bind hemoglobin or heme, since the majority of the protein incubated with heme- or hemoglobin-agarose was present in flow-through fractions (data not shown). These results further confirm that hemoglobin and heme binding to Kgp and HRgpA is mediated via the hemagglutinin domains of these proteins. The low binding of RgpB to hemoglobin and heme is most likely caused by the nonspecific tendency of heme to bind to most molecules and surfaces.

Interactions of gingipains with recombinant HmuR. In addition to binding hemoglobin, Kgp can degrade hemoglobin, as well as hemopexin, haptoglobin, and transferrin (6, 29, 53). Based on these results and the results presented here, we reasoned that in a soluble form Kgp could function as a heme-scavenging and hemoglobinase protein. The concept that Kgp could function in a multifactor manner in heme transport in *P. gingivalis* is supported by studies of the recently described *E. coli* hemoglobinase, Hbp (39). In addition to binding hemoglobin, Hbp can degrade hemoglobin and subsequently binds the released hemin. In a similar fashion, we postulate that Kgp could bind and degrade hemoglobin, and the released hemin could then be delivered to outer membrane receptor HmuR. This would require a direct interaction of Kgp with outer membrane receptor HmuR. To examine the possible association between Kgp or HRgpA and HmuR, we examined this interaction by an ELISA. The purity of rHmuR-6His preparation is shown in Fig. 4. Our analysis indicated that the gingipain preparations were pure and showed the same banding pattern after SDS-PAGE and CBB staining (Fig. 9A) as previously described (42, 44). This was also confirmed after transfer onto nitrocellulose membranes by probing with the appropriate antibodies raised to each gingipain (data not shown). As shown in Fig. 9B, rHmuR-6His was found to bind to Kgp. We also observed binding of rHmuR-6His to HRgpA at higher concentrations than those at which binding to Kgp was observed. We did not detect binding of rHmuR-6His to RgpB (Fig. 9B). We

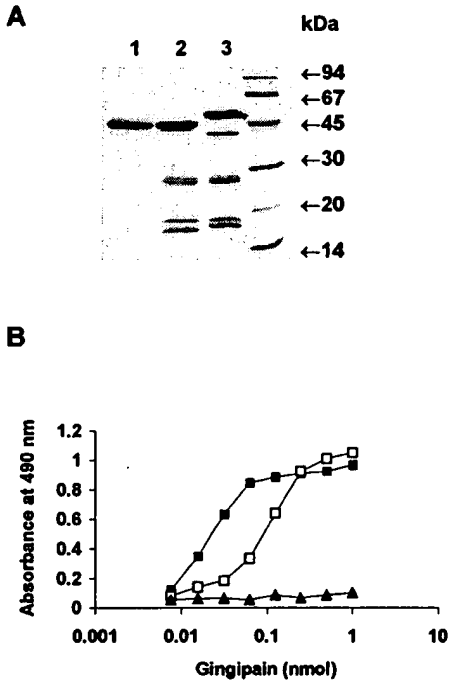

FIG. 9. Interaction between rHmuR-6His and gingipains. (A) SDS–12.5% PAGE gel stained with CBB showing the purity of gingipains RgpB (lane 1), HRgpA (lane 2), and Kgp (lane 3); molecular mass standards are shown on the right. (B) Reactivity of gingipains with soluble rHmuR-6His. Ni-NTA plates were coated with rHmuR-6His purified and renatured from inclusion bodies (0.05 nmol per well) and incubated with Kgp (■), HRgpA (□), or RgpB (▲) (0.005 to 1.0 nmol of gingipains per well). Binding was detected using antibodies to the appropriate gingipain. Values are representative of two separate experiments performed in triplicate.

also found that gingipain domains cleaved by boiling in the presence of SDS and then separated by SDS-PAGE and transferred onto nitrocellulose membrane failed to bind rHmuR-6His (data not shown). This suggests that the interaction between Kgp or HRgpA and HmuR requires the intact gingipain molecule but not denatured and separated gingipain domains.

DISCUSSION

In this study we assessed the binding specificities of the *P. gingivalis* outer membrane hemoglobin/heme receptor, HmuR, and cysteine proteases Kgp, HRgpA, and RgpB for hemoglobin, hemin, serum proteins, various porphyrins, and metalloporphyrins. While rHmuR-6His purified from inclusion bodies bound to hemin and hemoglobin and also, with lower affinity, to complexes of haptoglobin-hemoglobin, hemopexin-hemin, and albumin-hemin, we did not detect the binding of recombinant HmuR to apo-haptoglobin, albumin, apo-hemopexin, or transferrin saturated with iron. We also found that *E. coli* cells expressing rHmuR-6His bound not only hemin but also CuPPIX and ZnPPIX with similar affinities. These were bound approximately four times more tightly than PPIX itself. All three metals that we examined in this study (iron, copper, and zinc) can bind to the histidine within the active site in heme-proteins (1, 47, 55). The observation that these metalloprotoporphyrins bind to HmuR somewhat better than PPIX itself is consistent with a histidine in HmuR serving as the axial ligand, which binds to the metal ion present in the porphyrin ring. For the porphyrins themselves, we found that the order of affinity for HmuR-6His binding was as follows: MPIX > PPIX > DPIX ~ HPIX. This indicated that HmuR has a preference for ethyl or vinyl side chains of heme. In total, our results suggest that the HmuR binding site for heme has an axial histidine and accommodates a porphyrin structure with a periphery approximating that of the natural substrate hemin at the 2 and 4 positions. It should also be noted that differential-reconstitution experiments are subject to the issues that the porphyrins and metalloporphyrins may bind to other components in the mixture (20), that proteins and lipids interacting with one another may change the constant for binding to any individual component (8, 32), and that porphyrins and metalloporphyrins can aggregate in aqueous solution (7, 9, 27).

We demonstrated that soluble Kgp and HRgpA bound to the various porphyrins and metalloporphyrins with similar affinities. This indicates that any interaction between Kgp and HRgpA and the metal of the metalloporphyrin does not contribute significantly to recognition of the metalloporphyrin. Kgp and HRgpA bound hemoglobin more than 1 order of magnitude more tightly on a per-heme basis than any of the porphyrins and metalloporphyrins investigated. This is in agreement with a study by DeCarlo et al. (14), which reported that a recombinant polypeptide of the Kgp complex binds hemoglobin more efficiently than hemin. In that report the binding of the 19-kDa protein was inhibited by PPIX, indicating that hemin forms part of the recognition site for Kgp and hemoglobin. The results obtained in our study suggest that the recognition by Kgp and HRgpA of hemoglobin is mediated significantly via protein-protein interactions. Together these two studies point to roles for both heme-protein and protein-protein interactions in Kgp hemoglobin binding. Based on studies of two-component protein-protein complexes, it appears that heme itself will form only a minor portion of the recognition site, probably less than 20% (20, 30). The rest of the recognition could be due to the hemoglobin protein itself.

The dissociation constant of hemin binding to *E. coli* cells expressing recombinant HmuR is lower than K_ds for hemin receptors in *P. gingivalis* described by Tompkins et al. (54). According to this work *P. gingivalis* whole cells had both low- and high-affinity binding sites for hemin ($K_d = 2.6 \times 10^{-7}$ to 6.5×10^{-8} M and 3.6×10^{-11} to 9.6×10^{-11} M, respectively). Our K_d for HmuR expressed in *E. coli* (2.4×10^{-5} M) is similar to that found for the *S. marcescens* TonB-dependent hemoglobin/heme receptor, HasR ($K_d = 10^{-4}$ to 10^{-6} M) (21). In this study we have shown that *P. gingivalis* outer membrane receptor HmuR interacts with soluble gingipains Kgp and HRgpA. This suggests that Kgp and HRgpA might function as heme-scavenging proteins, cooperating with HmuR in hemoglobin and heme utilization in *P. gingivalis*. By analogy with the HasR-HasA system from *S. marcescens*, it is possible that HmuR requires a hemophore-like protein (Kgp or HRgpA) to increase hemoglobin and/or hemin binding to HmuR. Ghigo et al. (21) have shown that HasR alone is sufficient for hemoglobin and heme utilization but that *E. coli* more efficiently utilizes heme from hemoglobin via HasR-HasA cooperation ($K_d < 10^{-8}$ M). The HmuR system in *P. gingivalis* may function in an analogous manner with Kgp and HRgpA.

Okamoto et al. (38) previously reported that *P. gingivalis kgp* mutants are nonpigmented and are decreased in their ability to bind hemoglobin. The phenotype of the *kgp* mutants described by these investigators is similar to the phenotype of *P. gingivalis kgp* mutants, which we previously constructed and characterized (18; W. Simpson and C. A. Genco, unpublished data). These *P. gingivalis* mutant cells bind reduced levels of hemoglobin and hemin and exhibit a delayed growth with hemoglobin compared to the parental strain. We conclude from these results that Kgp may not be absolutely required for hemoglobin utilization in *P. gingivalis* but may make the process of hemoglobin utilization more efficient. We have previously reported that a *P. gingivalis hmuR* mutant does not grow with hemoglobin or hemin as the sole iron source, indicating that HmuR is required for hemoglobin utilization in *P. gingivalis* (50). We also observed that, following prolonged growth on blood agar plates, a *P. gingivalis hmuR* mutant is characterized by higher pigmentation capacity than wild-type *P. gingivalis* and nonpigmented *kgp* mutants (50; Simpson and Genco, unpublished data). This may be due to excessive heme storage, possibly through Kgp, and an inability to internalize heme due to the absence of HmuR. Furthermore, we found that a *P. gingivalis hmuR kgp* mutant is nonpigmented (Simpson and Genco, unpublished data), suggesting that this mutant cannot store and/or use heme due to the absence of HmuR and Kgp.

Overall our results indicate that outer membrane receptor HmuR exhibits higher specificity for heme than either Kgp or HRgpA. This finding would be expected if indeed soluble Kgp and HRgpA function more as heme scavenger proteins than as outer membrane receptors, which transport heme into the cell. It is noteworthy that the amino acid sequences of Kgp and HRgpA exhibit no similarity to those of TonB-dependent outer membrane proteins, further suggesting that these pro-

teins may function as extracellular heme-scavenging proteins rather than classical outer membrane receptors.

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Porphyrin-Mediated Binding to Hemoglobin by the HA2 Domain of Cysteine Proteinases (Gingipains) and Hemagglutinins from the Periodontal Pathogen *Porphyromonas gingivalis*

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Heme binding and uptake are considered fundamental to the growth and virulence of the gram-negative periodontal pathogen *Porphyromonas gingivalis*. We therefore examined the potential role of the dominant *P. gingivalis* cysteine proteinases (gingipains) in the acquisition of heme from the environment. A recombinant hemoglobin-binding domain that is conserved between two predominant gingipains (domain HA2) demonstrated tight binding to hemin (K_d = 16 nM), and binding was inhibited by iron-free protoporphyrin IX (K_i = 2.5 μM). Hemoglobin binding to the gingipains and the recombinant HA2 (rHA2) domain (K_d = 2.1 nM) was also inhibited by protoporphyrin IX (K_i = 10 μM), demonstrating an essential interaction between the HA2 domain and the heme moiety in hemoglobin binding. Binding of rHA2 with either hemin, protoporphyrin IX, or hematoporphyrin was abolished by establishing covalent linkage of the protoporphyrin propionic acid side chains to fixed amines, demonstrating specific and directed binding of rHA2 to these protoporphyrins. A monoclonal antibody which recognizes a peptide epitope within the HA2 domain was employed to demonstrate that HA2-associated hemoglobin-binding activity was expressed and released by *P. gingivalis* cells in a batch culture, in parallel with proteinase activity. Cysteine proteinases from *P. gingivalis* appear to be multidomain proteins with functions for hemagglutination, erythrocyte lysis, proteolysis, and heme binding, as demonstrated here. Detailed understanding of the biochemical pathways for heme acquisition in *P. gingivalis* may allow precise targeting of this critical metabolic aspect for periodontal disease prevention.

Evidence for the potential importance of cysteine proteinases from *Porphyromonas gingivalis* in periodontal disease pathology is increasing. Periodontal disease affects the majority of adults to some degree and may be associated with significant systemic morbidity (2, 46), including dental infection and loss of teeth (36). *P. gingivalis* is implicated as an important periodontal pathogen by its high incidence and relative levels in human disease (1, 11) and by its virulence in monoinfected animals (14, 15). Virulence of *P. gingivalis* has been attributed to several components of the microorganism, including fimbriae (25, 37), short-chain volatile acids (12, 65), lipopolysaccharide (26, 58), collagenase activity (3, 39), and noncollagenolytic cysteine proteinase activity (8, 10, 54).

Cysteine proteinase activity may affect the remodeling of matrix proteins and disrupt the immune response by stimulating the collagen-degrading activity of host cells (8, 10, 62), degrading fibronectin (34), inactivating gamma interferon (68) and interleukins (6, 17), interfering with the complement cascade (63, 67), and degrading immunoglobulins (16, 52). Also, clotting and vascular permeability mechanisms may be disturbed (27, 28, 54), fibrinogen may be degraded (33, 54), and erythrocytes may be agglutinated and lysed (44, 56) by cysteine proteinase activity, possibly for the acquisition of metabolically necessary iron, heme, or porphyrin from hemoglobin. Numerous different *P. gingivalis* cysteine proteinases described in several reports have been demonstrated to be antigenically related (9, 47, 48) and the products of three related genes (41, 51). This unique family of enzymes, named gingipains, has two major gene products, Arg-gingipain-1 (RGP-1) and Lys-gingipain (KGP) (41), which prefer proteinacious substrates with an arginine or lysine in the P1 position, respectively.

Bacterial cysteine proteinase activity has been demonstrated within diseased periodontal pockets (13, 20), and epitopes of gingipains are detectable in clinical plaque samples from patients with adult periodontitis (unpublished data), so the gingipains are likely to be clinically relevant. The gingipains are expressed on the outer membrane of *P. gingivalis* and may also be released with vesicles or as soluble proteins (9, 18, 24). Gingipains have been suggested to account for up to 85% of trypsin-like proteolytic activity in a *P. gingivalis* culture (49), and under certain growth conditions in vitro, these enzymes can accumulate to become the most abundant *P. gingivalis* proteins in a culture (9).

The catalytic domains of RGP-1 and KGP constitute approximately one-third of the translated protein products. The remaining two-thirds of these two gingipain molecules consist of four COOH-terminal domains (HA1 to HA4) which are highly homologous between these two predominant gingipains (Fig. 1). These noncatalytic COOH-terminal domains were originally named hemagglutinin (HA) domains because at least one was thought to participate in hemagglutination (47). They may each be separated posttranslationally from the catalytic domain and from one another, presumably through au-

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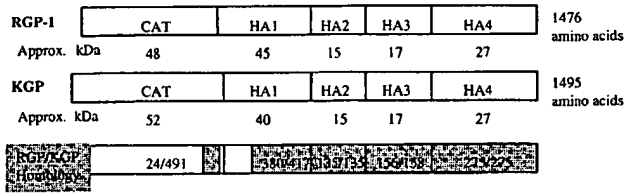

FIG. 1. Domain structure and homologies between the gingipains RGP-1 and KGP. CAT represents the putative catalytic domain. Shaded areas represent regions of >98% amino acid identity between the two gingipains. Each fraction represents the degree of identity for each RGP-1 domain. Approx. kDa, approximate molecular mass in kilodaltons.

tolysis some time after logarithmic growth in vitro (9, 59). The functions of the first, third, and fourth HA domains are unknown. The second HA domain (HA2) has recently been implicated in hemoglobin binding (19, 43). Because all of the domains of the gingipains are found together predominately in loose, noncovalent associations with one another after hydrolytic separation (9, 59), the gingipains appear to be multifunctional proteins for aggregation of erythrocytes and then lysing of these cells to obtain hemoglobin for the acquisition of iron, heme, or porphyrin.

P. gingivalis (formerly *Bacteroides* sp.) can utilize inorganic iron, free or protein-associated heme, or organic iron sources such as transferrin (5). Several investigators have previously shown that *P. gingivalis* binds to and internalizes hemin with various affinities and at various rates (4, 21, 53, 57, 60, 64). These earlier reports suggest that there are at least two heme-binding proteins of *P. gingivalis* with different affinities for hemin which may respond to environmental changes by rapidly changing their position or associations within the outer membrane.

Hemin binding and uptake appear to be related to the regulation of proteinase and fimbriae expression and to vesicle formation (7, 38, 40) and were recently proposed to establish an antioxidative shield for protection from oxidative radicals (61). Binding of protoporphyrin IX in *P. gingivalis* was also implied by competition with labelled hemin (4, 64), and protoporphyrin IX was reported to support growth (53). Protoporphyrin IX limitation was shown to be coordinated with phenotypic expression of proteinase activity (42). Hemin binding by *P. gingivalis* may therefore represent a capacity for protoporphyrin binding.

Recently, Nakayama et al. have isolated a hemoglobin-binding protein associated with the outer membrane of *P. gingivalis* and identified this protein as one homologous with the HA2 domain of the gingipains (43). In that report, adsorption of hemoglobin to whole *P. gingivalis* cells was associated with the presence of the HA2 domain. Also, hemin accumulation within the *P. gingivalis* cells was shown to be dependent on functional expression of KGP (45). The HA2 gingipain domain may therefore function as a hemoglobin-binding domain in *P. gingivalis*.

Understanding the molecular and biochemical mechanisms involved in key regulatory pathways is paramount in developing strategies for control of disease. In this study, we obtained evidence, by using a monoclonal antibody (MAb) which recognizes the hemoglobin-binding (HA2) domain of *P. gingivalis* cysteine proteinases, that the HA2 domain can bind to hemoglobin primarily and specifically through a portion of the heme moiety that is surface exposed in the hemoglobin structure. We also found that the unique epitope of MAb 5A1 within this heme-binding domain was expressed in parallel with hemoglobin-binding activity and proteinase activity in cellular and cell-free culture fractions of *P. gingivalis*.

MATERIALS AND METHODS

RGP-1 and KGP isolation. Polydomain RGP-1 and KGP were isolated and characterized as previously described (68) by arginine-Sepharose affinity chromatography of detergent-extracted *P. gingivalis* ATCC 33277 cells. Alternatively, polydomain RGP-1 and KGP were isolated as previously described (9) by arginine-Sepharose affinity chromatography from cell-free supernatant of a 10-day *P. gingivalis* batch culture.

Enzyme activity assays. The proteinase activities of *P. gingivalis* culture fractions were measured by using the substrates *N-tert*-butoxycarbonyl-Ile-Glu-Gly-Arg-7-amido-4-methylcoumarin or *N-tert*-butoxycarbonyl-Glu-Lys-Lys-7-amido-4-methylcoumarin at 30°C in Tris buffer without added reducing agents. Substrate hydrolysis was monitored over time by measuring A_{460} with a 380-nm excitation beam on a Perkin-Elmer LS 50B luminescence spectrophotometer.

Development of MAbs 5A1 and 1IB2. Antigingipain MAbs 5A1 and 1IB2 were prepared in mice as previously described (9).

Expression and purification of recombinant HA2 (rHA2). Forward and reverse primers (AACCTGCAGCGCGCAGACTTCACGG and GGAAGCCAA TGGCGCCAAAAGATCTAGT) were designed to amplify the HA2 domain from the *P. gingivalis* RGP-1 proteinase gene (accession no. U15282). Restriction sites for *Pst*I and *Bgl*II were designed into the 5′ ends of the primers to facilitate cloning. The digested PCR product was ligated into the QIAexpressionist type III construct providing a six-His tag at the COOH terminus (Qiagen Corp.). Transformation of the ligated construct was performed by electroporation into *Escherichia coli* NM522 cells. *E. coli* cultures were grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.6 and then induced by incubation with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h. Cells were harvested and resuspended to 5 ml/g (wet weight) in buffer A (8 M urea, 0.1 mM NaH_2PO_4, 0.01 mM Tris-HCl, pH 7.9). The cells were stirred for 2 h at room temperature, taking care to avoid foaming. This cell lysate was subjected to centrifugation at 31,000 × *g* for 30 min at room temperature to pellet the cellular debris, and then the supernatant was subjected to ultracentrifugation at 130,000 × *g* for 2 h. The clarified lysate was loaded onto a nickel-nitrilotriacetic acid column (Qiagen Corp.), pre-equilibrated with buffer A. The nickel-nitrilotriacetic acid column was washed with buffer A until the baseline was reached. The protein was refolded on this column by running a linear gradient of urea from 8 to 0 M in 20 mM Tris-HCl–500 mM NaCl–10% glycerol (pH 7.9). The protein was then eluted with 50 mM Tris-HCl–500 mM NaCl–10% glycerol–250 mM imidazole (pH 7.9). The eluant was diluted 100-fold in 50 mM sodium acetate buffer (pH 5.5) and applied to a hemoglobin-agarose column pre-equilibrated with the dilution buffer. After loading, the column was washed with the same buffer until the baseline was reached and then the hemoglobin-binding protein was eluted with 50 mM Tris-HCl (pH 9). Protein concentrations were determined by Coomassie dye binding using bovine serum albumin as the standard.

SDS-PAGE and Western blotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by using 12% gels with 4% stackers by the method of Laemmli (32). All samples were diluted with SDS sample buffer before electrophoresis with (reducing) or without 2-mercaptoethanol. Western blotting was performed by the method of Towbin et al. (66), and proteins were transferred from the gels to polyvinylidene difluoride (PVDF) paper (Bio-Rad) with 300 mA for 1 h. Blots were blocked with 0.1% bovine serum albumin in 20 mM Tris-HCl with 500 mM NaCl containing 0.1% Tween 20 (TBS/Tween). An alkaline phosphatase (AP) conjugate of rabbit anti-mouse immunoglobulin G (Dako Corp.) was used as a secondary antibody. Blots were washed with TBS/Tween between antibody applications. The substrate for AP was nitroblue tetrazolium in excess with 5-bromo-4-chloro-3-indolylphosphate (Bio-Rad), and color was developed in 5 mM Tris (pH 9.5).

NH_2-terminal amino acid sequencing of proteins resolved by SDS-PAGE was performed as previously described (9).

ELISA. Enzyme-linked immunosorbant assays (ELISA) were performed in polystyrene microtiter wells. Proteins were used to coat the surfaces in 2.7 mM KCl–1.5 mM KH_2PO_4–137 mM NaCl–8.1 mM Na_2HPO_4 (PBS) with 10 mM sodium azide (PBS/N_3). All wells were blocked and washed in PBS with 0.1% Tween 20 (PBS/Tween). Primary murine antibodies were applied in PBS/Tween at a concentration of 0.5 μg/ml for at least 1 h. Secondary goat anti-mouse antibodies conjugated with AP (Dako Corp.) were applied at a concentration of 1.1 μg/ml for 30 min, and then AP activity was monitored at 414 nm by hydrolysis of the substrate 4-nitrophenylphosphate (Boehringer GmbH, Mannheim, Germany) in 5 mM Tris (pH 9.5) by using a Titertek Twinreader PLUS photometer (absorbance maximum of 3.0 ELISA units). Mean apparent dissociation constants (K_ds) were derived by solid-phase ELISA as previously described (50) and are accompanied by standard errors of the means.

Ligand-binding assay. The ligand-binding assay was a variant of the ELISA in which the ligand (i.e., hemin or hemoglobin) that had been used to coat the wells in PBS/N_3 was subsequently allowed to bind to a second ligand-binding protein (i.e., rHA2 or gingipains) in PBS/Tween. The ligand-binding protein was then detected with MAb 5A1 or 1IB2, followed by a rabbit anti-mouse AP conjugate, and developed as already described for ELISA. Bovine hemoglobin was used in these experiments. Hemin was from stock solutions dissolved in 0.1 N NaOH,

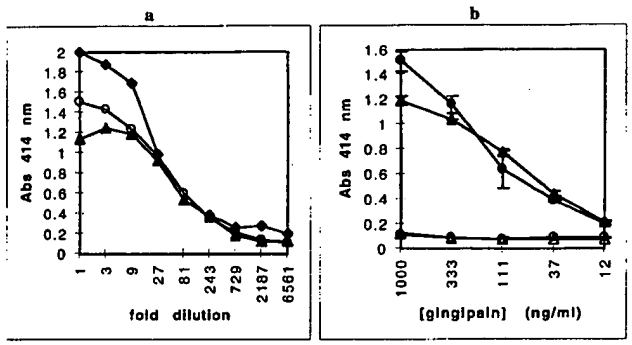

FIG. 2. Hemoglobin binding by rHA2, RGP-1, and KGP. (a) Microtiter wells were coated with hemoglobin and then incubated with threefold dilutions of purified rHA2 at 2.5 μg/ml (◆), RGP-1 at 5 μg/ml (○), or KGP at 5 μg/ml (▲). Association of rHA2 with hemoglobin was measured with MAb 5A1, followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody. (b) Hemoglobin binding by native, but not denatured, gingipains. Wells were coated with hemoglobin and then incubated overnight with dilutions of either RGP-1 (●), KGP (▲), RGP-1 denatured by boiling (○), or KGP denatured by boiling (△). For this experiment, native or denatured gingipains that bound to hemoglobin were recognized by MAb IIB2, which specifically detects both native and denatured gingipains. Primary antibody IIB2 was followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody. These data are representative of three separate experiments. Abs, absorbance.

and although the NaOH would replace the chloride ion of hemin with a hydroxylate ion (hematin), the term hemin will be used for this compound throughout this report. The K_d and apparent inhibition constant (K_i) for ligand binding were derived as previously described (50) in these assays by using serial dilutions of the ligand-binding protein or competitor, respectively, with even amounts of coated ligand. The reported results are means accompanied by the standard errors of the means.

Peptide synthesis. Peptides were synthesized by Chiron Mimotopes with terminal amines and carboxylic acids. The peptide 1 sequence was ALNPDNYLISKDVTG, and the peptide 2 sequence was GEAPAEWTTIDADGDGQGWL.

Materials. All chemicals and compounds were purchased from Sigma unless otherwise specified.

Statistics. Statistical differences between measurements of the gingipains and rHA2 were determined with one-tailed Student *t* tests.

RESULTS

The polydomain RGP-1 and KGP isolated from 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)-extracted *P. gingivalis* cells possessed SDS-PAGE profiles, NH_2-terminal sequences, proteolytic activities, and inhibition profiles characteristic of gingipain-like molecules previously described by us (9, 68) and others (3, 47, 54) (data not shown).

The HA2 domain was cloned, expressed, and purified as a six-His tag fusion. Nucleic acid and NH_2-terminal amino acid sequencing verified the identities of the clone and the expressed protein, respectively, as the HA2 domain of RGP-1 (data not shown).

Hemoglobin is bound by rHA2 and by native but not denatured RGP-1 and KGP. In the solid-phase ligand-binding assay, rHA2, RGP-1, and KGP each bound to hemoglobin (Fig. 2a). As MAb 5A1 was used to detect rHA2 bound to hemoglobin and did not interfere with this binding, it was evident that the epitope for MAb 5A1 within the HA2 domain was separate from the hemoglobin-binding site of HA2. The hemoglobin-binding affinities of rHA2, RGP-1, and KGP (K_d = 2.1 ± 0.6 nM) were similar (P = 0.24), and the binding curves of neither rHA2 nor the gingipains were indicative of multisite binding (Fig. 2a). High-affinity binding to hemoglobin at a single site within only the HA2 domain of both native RGP-1 and KGP is sufficient to account for these observations. The binding site for hemoglobin within the gingipains appeared to be associated with a higher-order protein structure, since denaturation of RGP-1 and KGP by boiling effectively eliminated their ability to bind hemoglobin (Fig. 2b).

Hemoglobin binding of the HA2 domain is mediated through the heme moiety. To begin characterizing the binding between rHA2 and hemoglobin, we examined the binding between rHA2 and hemin, as well as binding to hemoglobin degraded by proteinase K. rHA2 bound not only to wells coated with hemoglobin but also to wells coated with hemin or with proteolytically degraded hemoglobin (Fig. 3a). Binding of the rHA2 domain to hemin-coated wells was approximately eightfold weaker than binding to hemoglobin in solid-phase assays (K_d = 16 ± 1 nM) (Fig. 3b).

The HA2 domain binds the porphyrin ring structure. To dissect the binding of the rHA2 domain to hemin, the K_is of iron-free protoporphyrin IX in solution phase competition assays were determined. By using the standard ligand-binding assay described herein, rHA2 or the gingipains were preincubated with dilutions of protoporphyrin IX and then allowed to bind to the hemin-coated wells. Binding of the gingipains or rHA2 to hemin was inhibited by the addition of protoporphyrin IX (K_i = 2.5 ± 0.3 μM) (Fig. 4a). The apparent K_i values of rHA2 and the gingipains were similar (P = 0.42). These data indicated that binding of rHA2 or the gingipains to hemin was specific for some aspect of the protoporphyrin ring. Importantly, binding of rHA2 or the gingipains to hemoglobin was also inhibited by protoporphyrin IX (Fig. 4b) (K_i = 10 ± 2 μM) and preincubation with the protoporphyrin effectively eliminated binding to hemoglobin.

Directed protoporphyrin binding by rHA2. Examination of the hemoglobin crystal structure indicated that only the region of the heme moiety possessing the propionate functional groups (Fig. 5) would be exposed for possible protein-protein contact. We therefore reasoned that blocking access to the acidic region of protoporphyrin molecules would have an effect on rHA2 binding and allow more specific characterization of binding between the HA2 domain and the porphyrin ring. In a modification of the ligand-binding assay system described

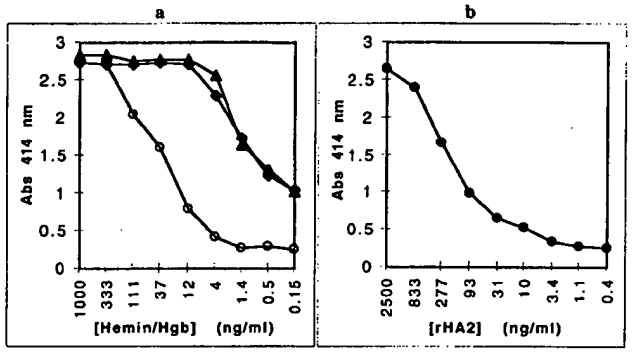

FIG. 3. Binding of the HA2 domain to the heme moiety. (a) Binding of rHA2 to dilutions of hemin (◆), hemoglobin (Hgb) (○), or hemoglobin degraded by proteinase K (▲). Microtiter wells were coated with dilutions of samples, and then overnight binding of rHA2 to coated wells was detected with MAb 5A1, followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody. The absence of contaminating protein within 90 μg of the hemin preparation and the absence of nondegraded subunits of hemoglobin remaining after proteinase K treatment were verified by SDS-PAGE (data not shown). (b) Binding of rHA2 to hemin. Microtiter wells were coated with hemin, and overnight binding of rHA2 dilutions was detected with MAb 5A1 as described above. These data are representative of two separate experiments. Abs, absorbance.

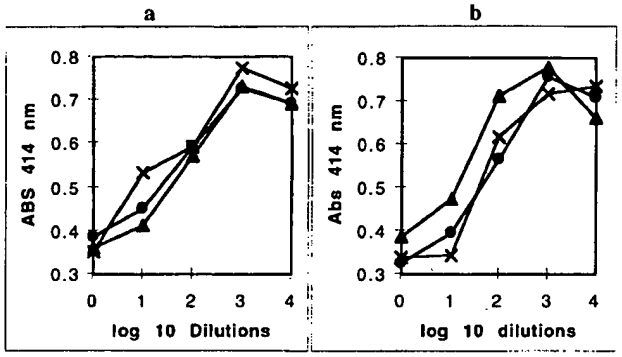

FIG. 4. Inhibition of hemin or hemoglobin binding. Microtiter wells were coated overnight with hemin (a) or hemoglobin (b). rHA2 in *E. coli* lysate (100-fold dilution) (×), RGP-1 at 65 ng/ml (●) or KGP at 65 ng/ml (▲) was preincubated with dilutions of 300 μM protoporphyrin IX for 1 h and then transferred to the ligand-coated plates for overnight incubation. Binding of rHA2 or the gingipains to coated wells was detected with MAb 5A1 or IIB2, respectively, followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody. These data are representative of two separate experiments. The absence of contaminating protein in a 90-μg protoporphyrin IX preparation was verified by SDS-PAGE and Coomassie dye binding (data not shown). ABS, absorbance.

above, surfaces were first coated with ethylene diamine to provide fixed, free, primary amines for carbodiimide linkage of carboxylic acid groups. Hemin, protoporphyrin IX, and hematoporphyrin bound to wells coated with ethylene diamine with or without carbodiimide treatment, as determined by A_{414} measurement (Fig. 5, striped bars). rHA2 binding to the carbodiimide-treated porphyrins in the wells was almost eliminated, however, compared to the relatively greater association of rHA2 with the nonderivatized porphyrins (Fig. 5, solid bars). These data indicated that the rHA2 domain specifically recognized the three porphyrin compounds in the region of the propionic acid groups, as we were able to block rHA2 binding by directionally attaching the carboxylic acids of hemin, protoporphyrin IX, or hematoporphyrin to fixed amines. Since the heme moiety within hemoglobin is almost identical to these porphyrin molecules, the data suggested that the heme moiety of hemoglobin was bound by rHA2 and by the HA2 domain of the gingipains in a similar, directed, high-affinity manner.

The MAb 5A1 epitope is recognized in the rHA2 domain and in denatured, but not native, RGP-1 and KGP. In ELISA, MAb 5A1 bound to rHA2 with high affinity (K_d = 2.2 × 10^{-10} ± 0.5 × 10^{-10} M) (Fig. 6a). MAb 5A1 also bound to denatured RGP-1 and KGP but did not bind to the native gingipains isolated from CHAPS-extracted *P. gingivalis* cells (Fig. 6b). Soluble high-molecular-weight aggregates of gingipain domains isolated from the cell-free fraction of a *P. gingivalis* batch culture by arginine-Sepharose affinity chromatography (9) were, however, recognized by MAb 5A1 (K_d = 1.7 × 10^{-10} ± 0.6 × 10^{-10} M) (Fig. 6c). The similarity of the dissociation constants (P = 0.36) and binding curves suggested that MAb 5A1 recognized the same HA2 epitope in these polydomain gingipains as in rHA2.

The MAb 5A1 epitope is represented by an amino acid sequence within the HA2 gingipain domain. By use of linear synthetic peptides, the epitope of MAb 5A1 was determined to be associated with the peptide ALNPDNYLISKDVTG (K_d = 3.8 nM), which represents amino acids 1215 to 1229 of translated KGP within the HA2 domain (Fig. 7, peptide 1). Dot blot analysis on a PVDF membrane confirmed the unique immunoreactivity of this peptide with MAb 5A1 (data not shown). A search of the SwissProt database for the linear sequence of peptide 1 or of the GenBank database by using the deduced nucleic acid sequence of this epitope resulted in no molecules with perfect homology to the peptide other than the gingipains and HagA, a large HA with regions of identity to the entire HA2 domain.

Correlation of HA2 domain immunoreactivity with hemoglobin binding in a *P. gingivalis* culture. Detection of the HA2 epitope with MAb 5A1 in unfractionated *P. gingivalis* samples was correlated with hemoglobin binding. Because proteinase activity and gingipain expression have been shown to progressively change during the course of an extended *P. gingivalis* batch culture (9), we examined cell-associated and extracellular fractions during 8 days of culture. Both Arg- and Lys-

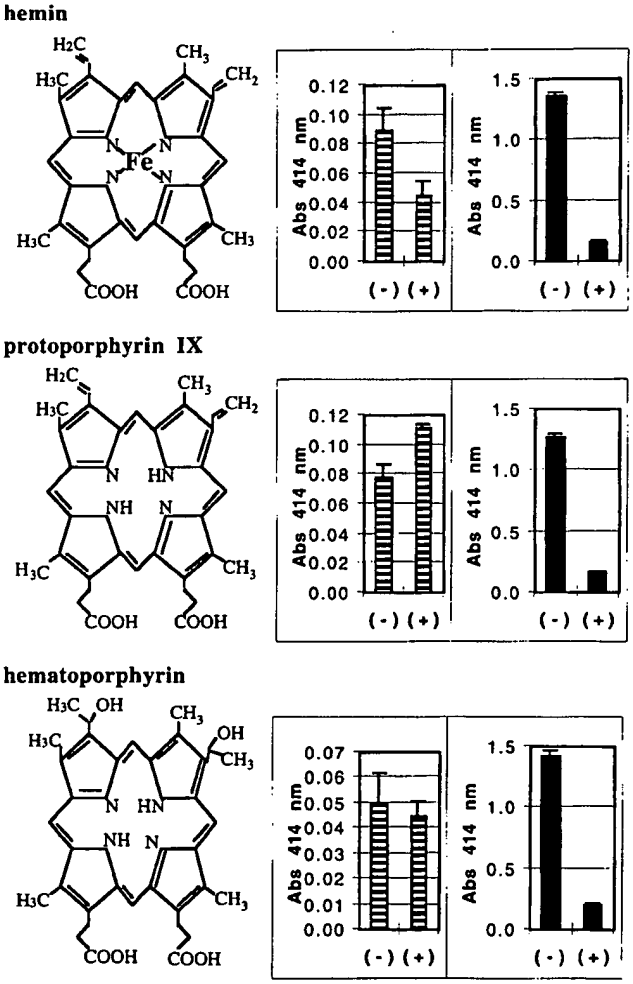

FIG. 5. Directed porphyrin binding by rHA2. Microtiter wells were coated with 100 mM ethylene diamine (pH 4.7) and then incubated with hemin, protoporphyrin IX, or hematoporphyrin at 90 μg/ml overnight in 50% dimethyl formamide in the presence (+) or absence (−) of 10 mM carbodiimide. Wells were washed four times with water, and then the amount of porphyrin bound to the wells was determined by measuring absorbance (Abs) at 414 nm (striped bars). Wells were blocked with PBS/Tween and then incubated with rHA2 at 125 ng/ml overnight. Binding of rHA2 to coated wells was detected with MAb 5A1, followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody (solid bars). Error bars represent standard deviations of absorbance measurements. Diagrams of the chemical structures of hemin, protoporphyrin IX, and hematoporphyrin are presented adjacent to the corresponding data.

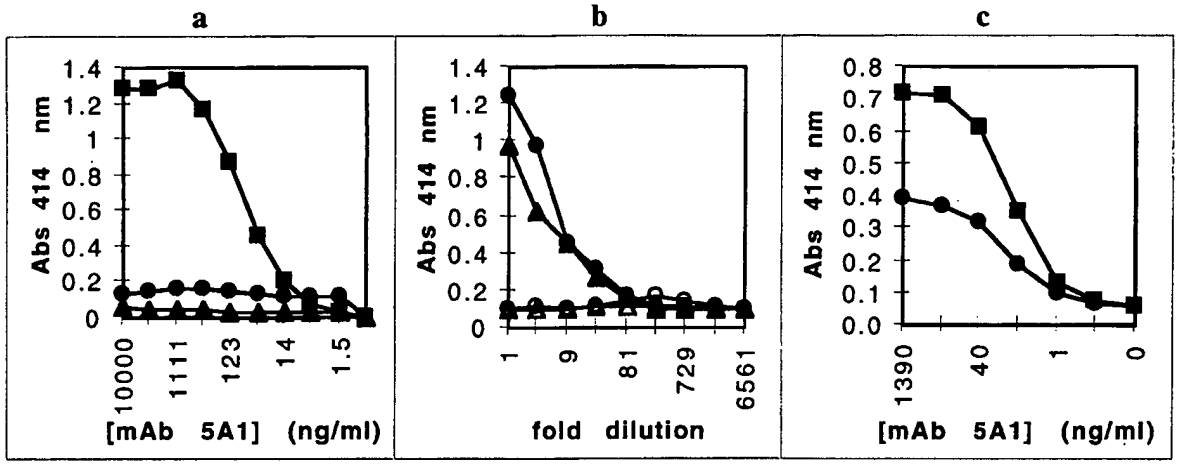

FIG. 6. Measurement of high-affinity binding of MAb 5A1 with rHA2, denatured but not native gingipains, and gingipains from the culture supernatant. (a) RGP-1 (●), KGP (▲), or rHA2 in crude *E. coli* lysate (■) was used to coat microtiter wells and incubated with serial dilutions of MAb 5A1. (b) Dilutions of RGP-1 (○), KGP (△), or heat-denatured RGP-1 (●) or KGP (▲) were used to coat microtiter wells with threefold dilutions from 10 μg/ml and then incubated with MAb 5A1. (c) Purified rHA2 (■) or purified high-molecular-weight aggregates of gingipain domains isolated from culture supernatant (●) were used to coat microtiter wells and incubated with serial dilutions of MAb 5A1. These data are representative of three separate experiments. Abs, absorbance.

specific proteinase activities of the *P. gingivalis* cells peaked near day 3 of culture (Fig. 8a and b, triangles). Proteinase activities of the cell-free culture supernatants steadily rose throughout the culture period (Fig. 8a and b, squares).

Immunoreactive protein in the cell-free conditioned culture medium detected with MAb 5A1 steadily accumulated throughout the 8-day culture period, similar to proteolytic activity (Fig. 8c, open squares). Immunoreactive protein associated with hemoglobin binding in this supernatant fraction also increased steadily throughout the extended culture in a parallel manner (Fig. 8c, closed squares). In the cellular fraction of the *P. gingivalis* culture, expression of immunoreactive protein increased early during the culture period with a peak near day 3 followed by a slight decrease and then an increase to peak levels again by day 7, similar to the proteolytic activity of this fraction (Fig. 8c, open triangles). Immunoreactive protein associated with hemoglobin binding in the cellular fraction followed a parallel pattern of expression (Fig. 8c, closed triangles). These data demonstrated that detection of protein immunoreactive with MAb 5A1 in crude cellular and extracellular fractions of a *P. gingivalis* culture was directly associated with hemoglobin binding, suggesting that MAb 5A1 specifically recognized the hemoglobin-binding HA2 domain within the *P. gingivalis* culture. Also, the data demonstrated a profile of HA2 domain expression and hemoglobin-binding activity similar to the profile of cellular and extracellular proteolytic activity expressed by *P. gingivalis*.

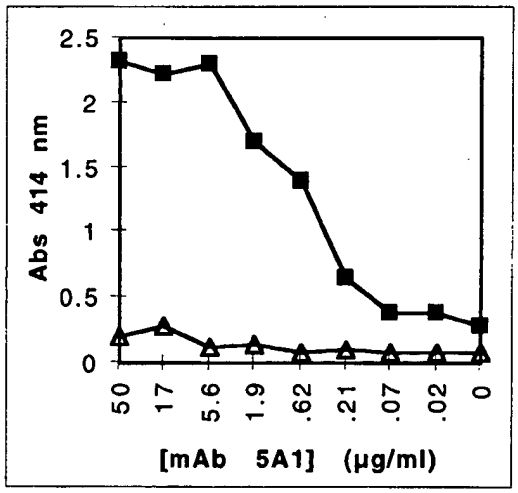

FIG. 7. Immunoreactivity of synthetic peptides with MAb 5A1. ELISA demonstrating selective immunoreactivity of MAb 5A1 with peptide 1. Peptide 1 (■) or 2 (△) was used to coat microtiter plates at a concentration of 5 μg/ml, incubated overnight, and then incubated with dilutions of MAb 5A1. These data are representative of two separate experiments. Abs, absorbance.

DISCUSSION

Control of *P. gingivalis* growth to prevent periodontal pathology might be achieved by interference with one or more pathways for obtaining heme. To this end, we have reported on a MAb which recognizes an epitope within the hemoglobin-binding domain of the abundant *P. gingivalis* cysteine proteinases, named gingipains, and demonstrated increasing levels of this HA2 domain associated with hemoglobin binding and proteinase activity in an extended *P. gingivalis* culture. Further, we have characterized the binding between the HA2 domain and hemoglobin, suggesting that binding is mediated in large part by specific recognition of the porphyrin ring of the heme moiety within hemoglobin.

The hemoglobin-binding affinities of RGP-1, KGP, and the HA2 domain measured in our experiments were similar. Also, binding curves for these interactions were typical of single-site binding, which is consistent with the idea that the HA2 domain of the cell-derived gingipains is solely responsible for hemoglobin binding. The similarity of the inhibition profiles for the gingipains to that of rHA2 further suggested that mediation of gingipain binding to heme was through only the HA2 domain. These data do not, however, rule out other possible heme-binding sites in the gingipains with affinity identical to that of HA2.

Hemoglobin binding by the separated catalytic domain of KGP was recently demonstrated (31). Our data, obtained by using polydomain gingipains, did not provide evidence for this

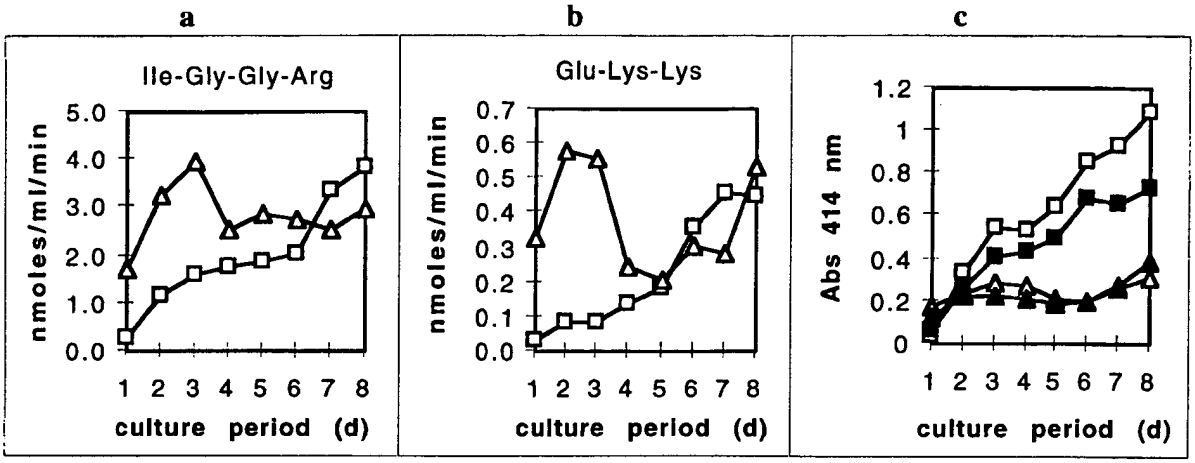

FIG. 8. Expression of HA2-related immunoreactive hemoglobin-binding protein from *P. gingivalis*. Aliquots of *P. gingivalis* culture medium were removed daily during a period of 8 days (d), immediately separated into a cell pellet and culture supernatant, and then frozen until use. The OD_{660} and purity of the culture were measured daily. The cell pellets were dispersed evenly into 1 ml of PBS/N_3. (a and b) Arg- and Lys-specific proteinase activities, respectively, of the cell-free culture supernatant (□) and cellular fraction (△) were measured as described in Materials and Methods. Measurements of the cellular fractions were normalized to the culture densities (OD_{660}) recorded daily. (c) The HA2 domain (1/243 dilution; □) and the HA2 domain associated with hemoglobin binding (1/81 dilution; ■) in culture supernatants were measured by ELISA and a ligand-binding assay, respectively, as described in Materials and Methods. In *P. gingivalis* whole-cell fractions, the HA2 domain (1/243 dilution; △) and the HA2 domain associated with hemoglobin binding (1/9 dilution; ▲) were measured by ELISA and a ligand-binding assay, respectively, as described in Materials and Methods. Measurements of the cell-associated fractions were normalized to the culture densities (OD_{660}) recorded daily. The corresponding background immunoreactivity with murine anti-human CD-19 immunoglobulin G was subtracted from each measurement. These data are representative of two separate experiments in which the patterns of expression were similar. Abs, absorbance.

second hemoglobin-binding site. It is likely, however, that separated domains of the gingipains behave differently than when associated either noncovalently or within a single polydomain polypeptide. The inability of MAbs which recognize either isolated gingipain domains or peptides to recognize the larger polydomain gingipains of cells exemplifies this potential (this report and reference 22).

Apparent dissociation constants in the nanomolar range represented significantly tighter binding of the HA2 domain to hemoglobin than previously reported (43). Further, this relatively tight binding in our experiments was measured at a nearly neutral pH and not at the pH maximum for binding of 5.5 reported earlier. Differences in experimental systems for measuring binding may account for this discrepancy.

Protoporphyrin IX inhibited binding of rHA2 to hemin. Also, protoporphyrin IX and hemin did not differ statistically in the ability to inhibit the binding of rHA2 to hemin (data not shown). This indicated that the sequestering of porphyrin by HA2 functioned independently of iron. The side chain groups of the porphyrin also did not appear to determine HA2 binding. Hematoporphyrin differs from protoporphyrin IX only by the hydroxylation of the two side chain ethylene groups. These groups are located opposite the positions of propionate groups across the plane of the porphyrin. As the binding to HA2 of these two porphyrins was comparably strong (Fig. 5), it can be concluded that HA2 binding was insensitive to the nature of the chemical groups attached at these positions. This contrasts with the blocking of rHA2 binding in both hematoporphyrin and protoporphyrin IX by directional attachment through chemical modification of the propionate groups.

The iron chelator 2,2′-dipyridal at a concentration of 2 mM also inhibited the binding of rHA2 to hemin, although the K_i of the dipyridal was 200-fold higher than the K_i of protoporphyrin IX (data not shown). This may indicate that rHA2 also had some weak interaction with the iron, but direct steric interference by the dipyridal in the absence of direct iron binding by rHA2 could also be considered.

Binding of hemin by the rHA2 domain was eightfold weaker than that of hemoglobin, although it would be expected to be similar if binding of the HA2 domain to hemoglobin occurred solely through the porphyrin ring of the heme ligand. Competition experiments demonstrated that protoporphyrin IX also inhibited hemoglobin binding, although it was approximately fourfold less competitive than in hemin-binding assays. A portion of the hemoglobin polypeptide may, therefore, contribute to the interaction of HA2 with hemoglobin in a cooperative manner. Because protoporphyrin IX alone completely blocked the interactions between rHA2 or the gingipains and hemoglobin, however, binding between the HA2 domain and the heme moiety must have been essential for the maintenance of this cooperative hemoglobin binding. Alternatively, the weaker binding of rHA2 with hemin in these experiments might also be due to the possibility that iron-protoporphyrins in solution can dimerize, ruffle, or associate differently than when bound to hemoglobin (23, 29, 55). Further, the HA2-binding region of the relatively smaller hemin ligand when bound directly to a surface may be less sterically accessible to the HA2 domain than when heme is presented and supported as part of a large globular protein where the propionate groups and the adjoining rim of the porphyrin ring protrude slightly beyond the surface of the protein (35).

Gingipains recovered from the culture supernatant subsequent to the first day of growth were previously shown to consist of noncovalently aggregated lower-molecular-weight domain fragments of the gingipains (9, 59). Although MAb 5A1 did not recognize native gingipains purified from solubilized *P. gingivalis* cells, MAb 5A1 did detect gingipain domain aggregates purified from the culture supernatant. This is not surprising, considering that the antibodies were made against the domain fragments of these gingipains (9), and it demonstrates potential differences between high-molecular-weight gingipains recovered by various means.

It is not known whether the HA2 domain was recognized in our cultures as a separate domain, as implicated by the isola-

tion of the separate HA2 domain from envelope fractions (19), or whether the HA2 domain was part of a polydomain complex of gingipain fragments or derived from the *hagA* gene product. Since the gingipains would be required for hydrolytic release of the HA2 domain from the *hagA* gene product, as well as from the gingipains themselves (43, 45), analysis of porphyrin binding in *hagA* knockout strains of *P. gingivalis* is needed to address this question. Our data demonstrated that the presence of the HA2 domain released by the cells paralleled proteinase activity, as well as hemoglobin-binding activity, suggesting that the hemoglobin-binding HA2 domain was derived from the gingipains. Although these data do not directly implicate the HA2 domain in iron, heme, or porphyrin acquisition by the *P. gingivalis* organism, the HA2 domain was associated with hemoglobin binding and could be considered a specific target for interference with heme acquisition by *P. gingivalis*. An HA2-specific antibody which blocks HA2 binding to heme or hemoglobin might be useful in dissecting the role of this porphyrin-binding domain in whole-cell metabolism and virulence.

Hemagglutination was the original function ascribed to the four COOH-terminal domains of the gingipains (47). Although the HA2 domain functions as a porphyrin-binding domain, it might, in addition, participate in hemagglutination. The separate rHA2 domain, at a concentration of 2 μg/ml, did not agglutinate erythrocytes, however, and MAb 5A1, which bound to the HA2 domain, did not inhibit the hemagglutination capacity of whole *P. gingivalis* cells (data not shown). We are currently investigating the functions of each gingipain HA domain.

Sequence analysis and trypsin susceptibility make the hemin-binding Omp26 described by Bramanti and Holt clearly different from the HA2 domain (4, 30). We have therefore identified a second hemin-binding protein in *P. gingivalis*. Interestingly, a recent independent analysis of hemin binding by whole cells of *P. gingivalis* described two different affinities (64). Now we have demonstrated that hemin- or hemoglobin-binding activity is also released by *P. gingivalis* in batch cultures. It is not immediately clear what advantage *P. gingivalis* would gain by releasing heme-binding activity, but it may be speculated, considering the recovery of the separate HA2 domain from the outer membrane (43), that soluble HA2 might reassociate with other gingipain domains on the *P. gingivalis* cells after scavenging and binding to heme or hemoglobin. A specific association of the HA2 domain with an active catalytic domain may be required for removal of the heme moiety from hemoglobin.

Characterization of the binding between the rHA2 domain and porphyrins should allow design of efficient affinity ligands for purification of HA2 and allow structure-based design of inhibitors of heme or hemoglobin binding. Heme acquisition is considered to be fundamental to the growth of *P. gingivalis*, and intervention with specific agents to disrupt pathways for heme binding or uptake may allow the eventual control or prevention of periodontal disease.

ACKNOWLEDGMENT

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